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(54) Title: OSTEONECTIN BASED TOXIC GENE THERAPY FOR THE TREATMENT OF CALCIFIED TUMORS AND TISSUES

(57) Abstract: The present invention relates to promoters, enhancers and other regulatory elements that direct expression within tumor and tissue cells with calcification potential. Specifically provided are expression vectors, host cells, and transgenic animals comprising an OSN regulatory region.

OSTEONECTIN BASED TOXIC GENE THERAPY FOR THE TREATMENT OF CALCIFIED TUMORS AND TISSUES

5 This application claims priority under 35 U.S.C. §119 (e) to U.S. provisional patent application no. 60/136,440 filed May 28, 1999, which is hereby incorporated by reference in its entirety.

1 INTRODUCTION

10 The present invention relates to promoters, enhancers and other regulatory elements that direct expression within tumor and tissue cells with calcification potential. In particular, it relates to compositions comprising nucleotide sequences from the 5' regulatory region, and transcriptionally active fragments thereof, that control expression of an osteonectin ("OSN"). Specifically provided are expression vectors, host cells and
15 transgenic animals wherein an OSN regulatory region is capable of controlling expression of a heterologous coding sequence, over-expressing an endogenous OSN coding sequence or an inhibitor of a pathological process or knocking out expression of a specific gene believed to be important for a calcification-related disease in tumor and tissue cells with calcification potential. The invention also relates to methods for using said vectors, cells and animals for
20 screening candidate molecules for agonists and antagonists of disorders involving tumor and tissue cells with calcification potential.

The present invention further relates to compositions and methods for modulating expression of compounds within tumor and tissue cells with calcification potential. The invention further relates to screening compounds that modulate expression
25 within tumor and tissue cells with calcification potential. Methods for using molecules and compounds identified by the screening assays for therapeutic treatments also are provided. The invention further relates to methods of treating tumors and other diseases and disorders involving tumor and tissue cells with calcification potential.

30 2 BACKGROUND OF THE INVENTION

2.1 Gene Therapy

Somatic cell gene therapy is a strategy in which a nucleic acid, typically in the form of DNA, is administered to alter the genetic repertoire of target cells for
35 therapeutic purposes. Although research in experimental gene therapy is a relatively young field, major advances have been made during the last decade. (Arai, Y., *et al.*, 1997,

Orthopaedic Research Society, 22:341). The potential of somatic cell gene therapy to treat human diseases has caught the imagination of numerous scientists, mainly because of two recent technologic advancements. Firstly, there are now numerous viral and non-viral gene therapy vectors that can efficiently transfer and express genes in experimental animals *in vivo*. Secondly, increasing support for the human genome project will allow for the identity and sequence of the estimated 80,000 genes comprising the human genome in the very near future.

Gene therapy was originally conceived of as a specific gene replacement therapy for correction of heritable defects to deliver functionally active therapeutic genes into targeted cells. Initial efforts toward somatic gene therapy relied on indirect means of introducing genes into tissues, called *ex vivo* gene therapy, e.g., target cells are removed from the body, transfected or infected with vectors carrying recombinant genes and re-implanted into the body ("autologous cell transfer"). A variety of transfection techniques are currently available and used to transfer DNA *in vitro* into cells; including calcium 15 phosphate-DNA precipitation, DEAE-Dextran transfection, electroporation, liposome mediated DNA transfer or transduction with recombinant viral vectors. Such *ex vivo* treatment protocols have been proposed to transfer DNA into a variety of different cell types including epithelial cells (U.S. Patent 4,868,116; Morgan and Mulligan WO87/00201; Morgan *et al.*, 1987, Science 237:1476-1479; Morgan and Mulligan, U.S. Patent No. 20 4,980,286), endothelial cells (WO89/05345), hepatocytes (WO89/07136; Wolff *et al.*, 1987, Proc. Natl. Acad. Sci. USA 84:3344-3348; Ledley *et al.*, 1987 Proc. Natl. Acad. Sci. 84:5335-5339; Wilson and Mulligan, WO89/07136; Wilson *et al.*, 1990, Proc. Natl. Acad. Sci. 87:8437-8441), fibroblasts (Palmer *et al.*, 1987, Proc. Natl. Acad. Sci. USA 84:1055-1059; Anson *et al.*, 1987, Mol. Biol. Med. 4:11-20; Rosenberg *et al.*, 1988, Science 242:1575-1578; Naughton & Naughton, U.S. Patent 4,963,489), lymphocytes (Anderson *et al.*, U.S. Patent No. 5,399,346; Blaese, R.M. *et al.*, 1995, Science 270:475-480) and 25 hematopoietic stem cells (Lim, B. *et al.* 1989, Proc. Natl. Acad. Sci. USA 86:8892-8896; Anderson *et al.*, U.S. Patent No. 5,399,346).

Direct *in vivo* gene transfer recently has been attempted with formulations of 30 DNA trapped in liposomes (Ledley *et al.*, 1987, J. Pediatrics 110:1), in proteoliposomes that contain viral envelope receptor proteins (Nicolau *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:1068) and DNA coupled to a polylysine-glycoprotein carrier complex. In addition, "gene guns" have been used for gene delivery into cells (Australian Patent No. 9068389). It even has been speculated that naked DNA, or DNA associated with liposomes, can be 35

formulated in liquid carrier solutions for injection into interstitial spaces for transfer of DNA into cells (Felgner, WO90/11092).

Numerous clinical trials utilizing gene therapy techniques are underway for such diverse diseases as cystic fibrosis and cancer. The promise of this therapeutic 5 approach for dramatically improving the practice of medicine has been supported widely, although there still are many hurdles that need to be passed before this technology can be used successfully in the clinical setting.

Perhaps, one of the greatest problems associated with currently devised gene therapies, whether *ex vivo* or *in vivo*, is the inability to control expression of a target gene 10 and to limit expression of the target gene to the cell type or types needed to achieve a beneficial therapeutic effect.

The concept of delivery and expression of therapeutic toxic genes to tumor cells through the use of tissue-specific promoters has been well recognized. This approach decreases the toxic effect of therapeutic genes on neighboring normal cells when vector 15 (virus, liposome, etc.) gene delivery results in the infection of the normal cells as well as the cancerous cells. Examples include the uses of α -fetoprotein promoter to target hepatoma cells (Koryama, *et al.*, 1991, Cell Struct. Punct., 16:503-510), the carcinoembryonic antigen (CEA) promoter for gastric carcinoma (Tanaka, *et al.*, 1996, Cancer Research, 46: 1341- 1345), the tyrosinase promoter to kill melanoma cells (Vile, *et al.*, 1994, Cancer Research, 20 54:6228-6234), the bone morphogenic protein promoter for brain to target glioma cells (Shimizu, K., 1994, Nippon Rinsbo, 52:3053-3058), and the osteocalcin promoter to kill osteocarcinoma and prostate cancers (Ko, S. *et al.*, 1996, Cancer Research, 56: 4614-4619; Gardener, *et al.*, 1998, Gene Therapy and Molecular Biology, 2:41-58). Molecular therapeutic strategies such as gene therapy through use of tissue and tumor-restricted 25 promoters are being used with increasing frequency. The key components of a gene therapy approach include: i) the selection of appropriate tissue-specific or tumor-restricted promoters, which, in some instances, may be inducible by a hormone, vitamin, an antibiotic, drug or heavy metal; ii) the selection of therapeutic (or toxic) genes; iii) the appropriate vectors, such as retrovirus, adenovirus, liposomes, etc. Key to targeting the appropriate 30 tumor tissue while sparing the normal host tissue is a promoter that can home the therapeutic genes to only those tissues which use the chosen promoter.

2.2 Tissue Specific Expression within Tumor and Tissue Cells with Calcification Potential

The treatment of osteotropic tumors such as breast, osteosarcoma and prostate which have metastasised is a major challenge. These seemingly unrelated diseases, 5 however, unite through a molecular analysis of the gene(s) that may be overexpressed in these forms of cancer during disease progression.

When evaluating human prostate cancer cells that have a propensity to metastasize to the skeleton, a surprising finding was that these cells have the ability to synthesize and secrete large amounts of non-collagenous bone matrix proteins, such as 10 osteopontin (OPN) (Thalman GN, *et al.*, 1997, Principles of Practice of Genitourinary Oncology, 409-416), osteocalcin (OC) (Curatolo C, *et al.*, 1992, European Urology, 1:105-107), and BSP (Withold W., *et al.*, 1997, Clinical Chemistry, 85-91).

OSN (also known as BM-40, basement membrane-40; SPARC, secreted protein acidic rich in Lysteine) is a 43 kDa protein highly conserved throughout species. 15 This protein is synthesized and secreted by some fetal and adult tissues and by a wide spectrum of human cancers. The gene is located at human chromosome 5q31-33. The size of the OSN gene is 26.5 kb with 10 exons separated by 9 introns. The OSN promoter was previously cloned and found to have no TATA or CAAT box. There are two GGA boxes in the human OSN promoter and three GGA boxes in the murine OSN promoter that are 20 important for transcriptional initiation. There is one major and one minor transcriptional start site that result in the transcription of a 2.2 and a 3.0 kb mRNA. Exon 1 of the OSN promoter is untranslated. Moreover, there is a repetitive CCTG element that was identified at exon 1, which is important for AP-2 transcriptional factor binding. The large intron 1 (>10 kb) contains numerous regulatory motifs, including retinoic acid, c-AMP, heat shock 25 proteins, metal ions and growth hormone.

OSN is involved in tumor invasion and metastasis. Through their interaction with OSN, tumor cells can be activated to secrete proteolytic enzymes to degrade the basement membrane. OSN-tumor interactions can result in the disruption of cellular adhesion, modulation of cellular proliferation, enhanced permeability and increased cell 30 migration and chemotaxis (Nature Med. 3:144, 1997). Other studies have demonstrated that OSN overexpression was found in most invasive malignant tumors, including those of mammary, ovarian, colon, squamous, endometrium, renal, hepatocellular, gall bladder, pulmonary and prostate cancers. In addition, several sarcomas, such as malignant fibrohistosarcoma, chondrosarcoma, angiosarcoma, hemangioendothelioma, osteosarcoma, 35 giant cell tumor of bone, neuroendocrine tumors such as carcinoids and small cell carcinoma of the lung, glioma, B cell lymphoma, seminoma, and melanoma also have

overexpression of OSN. In many situations, including that of the prostate, OSN overexpression correlates with neoplastic progression of breast cancer, colorectal cancer and myeloma. Further, it has been reported that suppression of OSN expression by antisense RNA strategy abrogates the tumorigenicity of human melanoma cells (Nature Med. 3:171, 5 1997).

3 SUMMARY OF THE INVENTION

The invention disclosed herein provides a model for osteotropic-specific gene transcription. The invention is based in part on the identification of a novel therapeutic agent for treating, curing and/or ameliorating tumors with calcification potential, including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, gastric, ovarian, and especially including, without limitation, breast and prostate cancers. The invention specifically targets sites of metastases of the above mentioned osteotropic tumors, and where applicable, their supporting osseous stroma in the metastatic environment. In addition, the present invention also relates to therapeutic agents which may also be applicable to benign conditions, such as benign prostatic hyperplasia (BPH) or arterial sclerotic conditions where calcification occurs.

The osteonectin ("OSN") promoter represents a novel sequence which has high activity in osteotropic tumors, and can be used as a powerful tool to direct the action of a chosen therapeutic gene to these osteotropic tumors in a tumor and tissue-restricted fashion. To date, the best studied therapeutic gene is herpes simplex virus thymidine kinase (HSVTK or TK) gene. Herpes simplex virus-TK converts the pro-drug ACV (or related drug) to a phosphorylated form that is cytotoxic to dividing cells (Moolten, F.L., 1996, Cancer Research, 46: 5276-5281). Critical to successful results is the "bystander" effect, which confers cytotoxicity on neighboring non-transduced cells; effective tumor cell kill can be achieved without the delivery to and expression of suicide genes in every tumor cell *in vivo*. This approach has been demonstrated recently to be efficacious in causing regression of many solid tumors in animal models (Tong, X.W. *et al.*, 1998, Anticancer Research, 18: 713-718). Often, in the recent literature, the vector for delivery of the chosen tumor-specific promoter and therapeutic (toxic) genes has been recombinant adenovirus containing the selected expression cassette, or a liposomal or retroviral formulation. A number of methods of vector delivery can be implemented, including injection, subcutaneous delivery, intraperitoneal delivery, intratumor delivery, intralesion delivery, intravenous delivery, intraosseous delivery, delivery by inhalation or loco-regionally by perfusion. Central to effective gene therapy is the choice of a tumor-specific promoter.

Because of the poor response rate of previously treated patients with relapsed prostate cancer (or other osteotropic tumors) to conventional radiotherapy, surgery, or chemotherapy, it is important to develop new therapeutic approaches that can be applied either independently or in conjunction with current or other novel treatment modalities. The 5 current invention provides the major advance of identifying a novel therapeutic gene that drives expression of therapeutic or toxic genes in a tumor and tissue-specific manner. More specifically, the instant invention provides, for the first time, *inter alia*, the identification of a novel therapeutic gene comprising the OSN promoter to direct osteotropic-specific expression, both *in vitro* in cultured osteotropic cells, and *in vivo* in transgenic animals.

10 The present invention provides a novel therapeutic composition comprising an OSN promoter that drives the expression of a therapeutic or toxic gene, for example herpes simplex virus thymidine kinase (TK) which is delivered by a vector, such as a recombinant adenovirus (Ad), to a variety of human tumors or benign tissues that exhibit the ability to calcify either in the primary or at metastatic sites. This is especially evident 15 for prostate and osteosarcoma tumors, but includes any osteotropic aggressive metastatic tumor such as, for example, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, gastric, ovarian, and especially including, without limitation, breast and prostate cancers. Non-tumor cells which have the ability to calcify and thus express OSN also are able to express high levels of the recombinant 20 reporter or therapeutic genes of the present invention.

The present invention also provides a method of treating osteosarcoma or prostate cancer or other osteotropic tumors that are able to use the OSN promoter by the above routes of recombinant adenovirus Ad-OSN-TK administration in combination with a prodrug, most commonly acyclovir (ACV), although the OSN promoter-driven therapy is 25 not limited to a specific vector or therapeutic gene. Indeed, conceivably, many forms of vector or toxic gene can be generated and combined with this novel OSN promoter-driven strategy to obtain a desired antitumor effect. Based upon the level and tissue-specificity of OSN promoter expression, when combined with a recombinant vector and toxic or therapeutic gene, the present invention will effectively eliminate prostate and osteosarcoma 30 or other osteotropic tumors, including, but not limited to, lung, colon, melanoma, thyroid, brain, multiple myeloma, and breast cancers growth both *in vitro*, and *in vivo* as localized and as osseous metastatic deposits.

The present invention provides compositions and methods for screening compounds that modulate expression within osteotropic cells and tissues. In particular, it 35 provides compositions comprising nucleotides from the human OSN promoter, and transcriptionally active fragments thereof, as well as nucleic acids that hybridize under

highly stringent conditions to such nucleotides, that control the expression of an osteotropic-specific gene. Specifically provided are expression vectors comprising the OSN promoter, and transcriptionally active fragments thereof, operably associated to a heterologous reporter gene, *e.g.*, luciferase, and host cells and transgenic animals containing such vectors. The invention also provides methods for using such vectors, cells and animals for screening candidate molecules for agonists and antagonists of osteotropic-related disorders. Methods for using molecules and compounds identified by the screening assays for therapeutic treatments also are provided.

For example, and not by way of limitation, a composition comprising a reporter gene is operatively linked to an OSN promoter. The OSN driven reporter gene is expressed as a transgene in animals. The transgenic animal, and cells derived from osteotropic cells of such transgenic animal, can be used to screen compounds for candidates useful for modulating osteotropic-related disorders. Without being bound by any particular theory, such compounds are likely to interfere with the function of trans-acting factors, such as transcription factors, cis-acting elements, such as promoters and enhancers, as well as any class of post-transcriptional, translational or post-translational compounds involved in osteotropic-related disorders. As such, they are powerful candidates for treatment of such disorders, including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, gastric, ovarian, and especially including, without limitation, breast and prostate cancers, and benign conditions, such as BPH or arterial sclerotic conditions where calcification occurs. The compounds of the invention additionally can be used to express crucial growth and differentiation associated genes such as growth factors, growth factor receptors, bone morphogenic proteins, *etc.*, for repairing the damages acquired during aging and degenerative conditions.

In one embodiment, the invention provides methods for high throughput screening of compounds that modulate specific expression of genes within osteotropic cells and tissues. In this aspect of the invention, cells from osteotropic-tissues are removed from the transgenic animal and cultured *in vitro*. The expression of the reporter gene is used to monitor osteotropic-specific gene activity. In a specific embodiment, luciferase is the reporter gene. Compounds identified by this method can be tested further for their effect on osteotropic-related disorders in normal animals.

In another embodiment, the transgenic animal models of the invention can be used for *in vivo* screening to test the mechanism of action of candidate drugs for their effect on osteotropic-related disorders. Specifically, the effects of the drugs on osteotropic-related disorders including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, gastric, ovarian, and especially including,

without limitation, breast and prostate cancers, and benign conditions, such as BPH or arterial sclerotic conditions where calcification occurs, can be assayed.

In another embodiment, a gene therapy method for treating and/or preventing osteotropic-related disorders is provided. OSN promoter sequences are used to drive 5 osteotropic-specific expression of toxic or therapeutic molecules and introduced in the osteotropic cells. The method comprises introducing an OSN promoter sequence operatively associated with a nucleic acid encoding a toxic or therapeutic molecule into osteotropic cells. In one embodiment, the invention provides a preventative gene therapy method comprising introducing an OSN promoter sequence operatively associated with a 10 nucleic acid encoding a toxic or therapeutic molecule into osteotropic cells to delay and/or prevent an osteotropic-related disorder. In a specific embodiment, the invention provides a gene therapy method for treatment of cancer or other proliferative disorder, including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, gastric, ovarian, and especially including, without limitation, 15 breast and prostate cancers. The OSN promoter sequence is used to direct the expression of one or more proteins specifically in the osteotropic-tumor cells of a patient. In addition, due to the tissue specificity of the promoters used in the present invention, therapeutic and/or toxic agents are effective not only when administered via direct application, such as by injection, but also when administered systemically to the body via intravenous 20 administration, intra-arterial administration, intra-tumoral administration, perfusion, oral administration or the like, because gene expression will be limited and localized to specific cell and tissue types. Further, since many of the therapeutic and/or toxic agents of the invention exhibit pleiotropic effects, expression of the therapeutic and/or toxic agents in only specifically targeted cells is essential in order to prevent numerous, harmful side 25 effects.

In addition to tissue specific promoters, the present invention encompasses vectors using inducible promoters. Inducible promoters have the advantage that they can be switched on and off, depending on the clinical state of the patient. Therefore, if a cell is stably transfected with a therapeutic transgene under the control of an inducible promoter, 30 its expression could be controlled over the life-time of an individual.

The invention further provides methods for screening for novel transcription factors that modulate the OSN promoter sequence. Such novel transcription factors identified by this method can be used as targets for treating osteotropic-related disorders.

3.1 Definitions

	TK	= thymidine kinase;
	OC	= osteocalcin;
	OSN	= osteonectin;
5	AcV	= acyclovir;
	FBS	= fetal bovine serum;
	Beta-gal.	= beta-galactosidase;
	CMV	= cytomegalovirus;
	ROS 17/2.7	= rat osteoblastic osteosarcoma;
10	MG 63	= human osteosarcoma;
	NIH 3T3	= embryonic mouse fibroblast;
	P69	= human "normal" prostate cell type without any tumorogenic or metastatic ability;
	LNCaP	= human androgen dependent prostate cancer;
15	C4-2	= human androgen independent highly tumorogenic/metastasizing prostate cancer;
	PC-3M	= human androgen independent highly metastatic prostate cancer;
	ArCaP	= human androgen independent prostate cancer;
20	Saos-2	= human osteosarcoma;
	SF/PF	= serum free, phenol free
	DI	= mouse embryonic pluripotent bone marrow cell;
	Lovo	= human colon cancer;
	MCF-7	= human breast cancer;
25	U-97	= human brain cancer of the glioblastoma multiform type;
	A547	= human lung cancer;
	DMEM	= Dulbecco's Modified Eagle Media;
	T media	= prostate cancer cell optimal growth media;
	RLU	= relative luciferase units;

30

4 BRIEF DESCRIPTION OF THE FIGURES

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be understood better by reference to one or more of these drawings in combination with the 35 detailed description of specific embodiments presented herein.

Figure 1: OSN Regulatory Region Sequence from -1409 to +904.

5 **Figure 2:** Schematic Representation of the Strategy used to Construct OSN deletion constructs.

Figure 3: Promoter Activity of OSN Deletion Constructs in Prostatic and Non-prostatic Cell Lines.

10 **Figure 4:** Western Blot Revealing OSN Protein Expression in various Prostatic and Non-prostatic Cell Lines.

15 **Figure 5:** Northern Blot Revealing OSN Expression (ON) in Various Prostate Cancer Cell Lines.

20 **Figure 6:** Northern Blot Revealing OSN Expression (ON) in Various Prostate Cancer Tissues.

Figure 7: Schematic Representation of a 2.3 kb Human Osteonectin Gene Promoter.

25 **Figure 8:** Promoter Activity of OSN Deletion Constructs in Various Cancer Cell Lines.

Figure 9: Comparison of OSN Promoter Activity in Various Cancer Cell Lines.

30 **Figure 10A-10B:** *In Vitro* Cytotoxicity Assay with AD-522E-TK. Figure 10A represents the assay with PC3M cells. Figure 9B represents the assay with MG63 cells.

Figure 11: OSN Regulatory Region Sequence from -522 to +62.

35 **Figure 12:** PC3M Subcutaneous Tumor Growth in Athymic Mice Injected with Ad-522E-TK.

5 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides promoters, enhancers and other regulatory elements that direct expression within osteotropic cells, comprising nucleotide sequences from the 5' regulatory region, and transcriptionally active fragments thereof, that control expression of an OSN. Specifically provided are expression vectors, host cells and transgenic animals wherein an OSN regulatory region is capable of controlling expression of a heterologous coding sequence, over-expressing an endogenous OSN gene or an inhibitor of a pathological process or knocking out expression of a specific gene believed to be important for a calcification-related disease in tumor and tissue cells with calcification potential.

The invention also provides methods for using said vectors, cells and animals for screening candidate molecules for agonists and antagonists of disorders involving tumor and tissue cells with calcification potential. In an alternate embodiment, the invention provides compositions and methods for modulating expression of compounds within tumor and tissue cells with calcification potential, and to screening compounds that modulate expression within tumor and tissue cells with calcification potential. Methods for using the molecules and compounds identified by the screening assays for therapeutic treatments also are provided.

The invention further provides methods of treating and/or ameliorating tumors and other diseases and disorders with calcification potential, including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, breast and prostate cancers. The invention specifically targets sites of metastases of the above mentioned osteotropic tumors, and where applicable, their supporting osseous stroma in the metastatic environment. In addition, the present invention provides therapeutic agents which may be applicable to benign conditions such as benign prostatic hyperplasia (BPH) or arterial sclerotic conditions where calcification occurs.

The invention is based, in part, on the discovery that nucleotide sequences encoding toxic and/or therapeutic coding sequences contained within vectors (*i.e.* viral vectors) can be administered in a cell and tissue specific manner, with the use of promoters which allow for tissue specific expression of the nucleotide sequences. Further, because the vectors of the invention utilize these promoters to control the expression of toxic and/or therapeutic coding sequences, the vectors of the invention are effective therapeutic agents not only when administered via direct application, but also when administered systemically to the body, because the toxic and/or therapeutic coding sequences will be expressed only in specifically targeted cells, *i.e.*, within tumor and tissue cells with calcification potential.

Taking advantage of this feature, the methods of the present invention are designed to efficiently transfer one or more DNA molecules encoding therapeutic agents to a site where the therapeutic agent is necessary. The methods involve the administration of a vector containing DNA encoding translational products (*i.e.* therapeutic proteins) or transcriptional products (*i.e.* antisense or ribozymes) within a mammalian host to a site where the translational product is necessary. Once the vector infects cells where the therapeutic agent is necessary, the coding sequence of interest, *i.e.*, thymidine kinase, is expressed, thereby amplifying the amount of the toxic and/or therapeutic agent, protein or RNA.

10 The present invention relates also to pharmaceutical compositions comprising vectors containing DNA for use in treating and/or ameliorating osteotropic-related disorders, including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, breast and prostate cancers, and benign conditions, such as, for example, benign prostatic hyperplasia (BPH) or arterial

15 sclerotic conditions where calcification occurs. The compositions of the invention generally are comprised of a bio-compatible material containing the vector containing DNA encoding a therapeutic protein of interest, *i.e.*, thymidine kinase, growth factors, etc. A bio-compatible composition is one that is in a form that does not produce an allergic, adverse or other untoward reaction when administered to a mammalian host.

20 The invention overcomes shortcomings specifically associated with current recombinant protein therapies for treating and/or ameliorating osteotropic diseases. First, direct gene transfer is a rational strategy that allows transfected cells to (a) make physiological amounts of therapeutic protein, modified in a tissue- and context-specific manner, and (b) deliver this protein to the appropriate cell surface signaling receptor under

25 the appropriate circumstances. Exogenous delivery of such molecules is expected to be associated with significant dosing and delivery problems. Second, repeated administration, while possible, is not required with the methods of the invention because various promoters, including inducible promoters, can be used to control the level of expression of the therapeutic protein of interest. Further, integration of transfected DNA can be associated with long term recombinant protein expression.

30 Described in detail below, in Sections 5.1 and 5.2, are nucleotide sequences of the OSN regulatory region, and expression vectors, host cells and transgenic animals wherein the expression of a heterologous coding sequence is controlled by the OSN regulatory region. In Section 5.3, methods for using such polynucleotides (*i.e.*, regulatory regions of the OSN gene) and fusion protein products, for screening compounds that interact

with the regulatory region of the OSN gene are described. This Section describes both *in vivo* and *in vitro* assays to screen small molecules, compounds, recombinant proteins, peptides, nucleic acids, antibodies, etc. which bind to or modulate the activity of the OSN regulatory region. Section 5.4 describes methods for the use of the compositions of the invention, identified agonists and antagonists for drug delivery or gene therapy. Finally, in Section 5.5, pharmaceutical compositions are described for using such compositions, agonists and antagonists to modulate osteotropic-related disorders. Methods and compositions are provided for treating various osteotropic-related disorders, including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, breast and prostate cancers, and benign conditions, including, but not limited to, benign prostatic hyperplasia (BPH) or arterial sclerotic conditions where calcification occurs.

5.1 Polynucleotides and Nucleic Acids of the Invention

The present invention encompasses polynucleotide sequences comprising the 5' regulatory region, and transcriptionally active fragments thereof, of the OSN gene. In particular, the present invention provides polynucleotides comprising about 2.3 kb, 1.5 kb, 1.1 kb, 0.6 and 0.2 kb sequences that are located within an OSN gene. Specifically, the polynucleotides comprise -1409 bp through +904 bp, -1409 bp through +73 bp, -120 bp through +904 bp and -120 bp through +73 bp of the OSN sequence shown in Figure 1 and -522 bp through +39, -522 bp through +62 and -522 bp through +73 of the OSN sequence shown in Figure 11. In various embodiments, the polynucleotide may be 5000, 4000, 3000, 2000, 1000 and preferably approximately 500 bp in length.

The invention further provides probes, primers and fragments of the OSN regulatory region. In one embodiment, purified nucleic acids consisting of at least 8 nucleotides (*i.e.*, a hybridizable portion) of an OSN gene sequence are provided; in other embodiments, the nucleic acids consist of at least 20 (contiguous) nucleotides, 25 nucleotides, 50 nucleotides, 100 nucleotides, 200 nucleotides, 500, 1000, 2000, 3000, 4000 or 5000 nucleotides of an OSN sequence. Methods which are well known to those skilled in the art can be used to construct these sequences, either in isolated form or contained in expression vectors. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* genetic recombination. See, *e.g.*, the techniques described in Sambrook *et al.*, 1989, *supra*, and Ausabel *et al.*, 1989, *supra*; also see the techniques described in "Oligonucleotide Synthesis", 1984, Gait M.J. ed., IRL Press, Oxford, which is incorporated herein by reference in its entirety.

In another embodiment, the nucleic acids are smaller than 20, 25, 35, 200 or 500 nucleotides in length. Nucleic acids can be single or double stranded. The invention also encompasses nucleic acids hybridizable to or complementary to the foregoing sequences. In specific aspects, nucleic acids are provided which comprise a sequence 5 complementary to at least 10, 20, 25, 50, 100, 200, 500 nucleotides or the entire regulatory region of an OSN gene.

The probes, primers and fragments of the OSN regulatory region provided by the present invention can be used by the research community for various purposes. They can be used as molecular weight markers on Southern gels; as chromosome markers or tags 10 (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in patients to identify potential genetic disorders; as probes to hybridize and thus discover novel, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; and as a probe to "subtract-out" known sequences in the process of discovering other novel polynucleotides. Methods for 15 performing the uses listed above are well known to those skilled in the art. References disclosing such methods include, without limitation, "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E. F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S. L. and A. R. Kimmel eds., 1987.

20 The nucleotide sequences of the invention also include nucleotide sequences that have at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more nucleotide sequence identity to the nucleotide sequence depicted in Figure 1, and/or transcriptionally active fragments thereof, which are capable of driving expression specifically within tumor and tissue cells with calcification potential.

25 To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then 30 compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical overlapping positions/total # of positions x 100). In one embodiment, the two 35 sequences are the same length.

The determination of percent identity between two sequences also can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin 5 and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with 10 the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an 15 iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used (see <http://www.ncbi.nlm.nih.gov>). Another preferred, non-limiting example of a mathematical algorithm utilized for the 20 comparison of sequences is the algorithm of Myers and Miller, (1988) *CABIOS* 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 can be used. In an alternate embodiment, alignments can be obtained using the NA_MULTIPLE_ALIGNMENT 1.0 program, using a GapWeight of 5 25 and a GapLengthWeight of 1.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

The invention also encompasses:

- 30 (a) DNA vectors that contain any of the foregoing OSN regulatory sequences and/or their complements (*i.e.*, antisense);
- (b) DNA expression vectors that contain any of the foregoing OSN regulatory element sequences operatively associated with a heterologous gene, such as a reporter gene; and

(c) genetically engineered host cells that contain any of the foregoing OSN regulatory element sequences operatively associated with a heterologous gene such that the OSN regulatory element directs the expression of the heterologous gene in the host cell.

Also encompassed within the scope of the invention are various transcriptionally active fragments of this regulatory region. A "transcriptionally active" or "transcriptionally functional" fragment of the sequence depicted in Figure 1 according to the present invention refers to a polynucleotide comprising a fragment of said polynucleotide which is functional as a regulatory region for expressing a recombinant polypeptide or a recombinant polynucleotide in a recombinant cell host. For the purpose of the invention, a nucleic acid or polynucleotide is "transcriptionally active" as a regulatory region for expressing a recombinant polypeptide or a recombinant polynucleotide if said regulatory polynucleotide contains nucleotide sequences which contain transcriptional information, and such sequences are operably associated to nucleotide sequences which encode the desired polypeptide or the desired polynucleotide.

In particular, the transcriptionally active fragments of the OSN regulatory region of the present invention encompass those fragments that are of sufficient length to promote transcription of a heterologous gene, such as a reporter gene, when operatively linked to the OSN regulatory sequence and transfected into tumor and tissue cells with calcification potential. Typically, the regulatory region is placed immediately 5' to, and is operatively associated with the coding sequence. As used herein, the term "operatively associated" refers to the placement of the regulatory sequence immediately 5' (upstream) of the reporter gene, such that trans-acting factors required for initiation of transcription, such as transcription factors, polymerase subunits and accessory proteins, can assemble at this region to allow RNA polymerase dependent transcription initiation of the reporter gene.

In one embodiment, the polynucleotide sequence chosen may further comprise other nucleotide sequences, either from the OSN gene, or from a heterologous gene. In another embodiment, multiple copies of a promoter sequence, or a fragment thereof, may be linked to each other. For example, the promoter sequence, or a fragment thereof, may be linked to another copy of the promoter sequence, or another fragment thereof, in a head to tail, head to head, or tail to tail orientation. In another embodiment, an osteotropic-specific enhancer may be operatively linked to the OSN regulatory sequence, or fragment thereof, and used to enhance transcription from the construct containing the OSN regulatory sequence.

Also encompassed within the scope of the invention are modifications of this nucleotide sequence without substantially affecting its transcriptional activities. Such modifications include additions, deletions and substitutions. In addition, any nucleotide

sequence that selectively hybridizes to the complement of the sequence depicted in Figure 1 under stringent conditions, and is capable of activating the expression of a coding sequence specifically within tumor and tissue cells with calcification potential is encompassed by the invention. Exemplary moderately stringent hybridization conditions are as follows:

- 5 prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 hours at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10⁶ cpm of ³²P-labeled probe. Washing of filters is done at 37°C
- 10 for 1 hour in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Alternatively, exemplary conditions of high stringency are as follows: e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current
- 15 Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3). Other conditions of high stringency which may be used are well known in the art. In general, for probes between 14 and 70 nucleotides in length the melting temperature (Tm) is calculated using the formula:
$$Tm(^{\circ}C)=81.5+16.6(\log[\text{monovalent cations (molar)}])+0.41 (\% G+C)-(500/N)$$
 where N is
- 20 the length of the probe. If the hybridization is carried out in a solution containing formamide, the melting temperature is calculated using the equation
$$Tm(^{\circ}C)=81.5+16.6(\log[\text{monovalent cations (molar)}])+0.41(\% G+C)-(0.61\% \text{formamide})-(500/N)$$
 where N is the length of the probe. In general, hybridization is carried out at about 20-25 degrees below Tm (for DNA-DNA hybrids) or 10-15 degrees below Tm (for RNA-DNA hybrids).

The OSN regulatory region, or transcriptionally functional fragments thereof, is preferably derived from a mammalian organism. Screening procedures which rely on nucleic acid hybridization make it possible to isolate gene sequences from various organisms. The isolated polynucleotide sequence disclosed herein, or fragments thereof, 30 may be labeled and used to screen a cDNA library constructed from mRNA obtained from appropriate cells or tissues (e.g., calcified tissue) derived from the organism of interest. The hybridization conditions used should be of a lower stringency when the cDNA library is derived from an organism different from the type of organism from which the labeled sequence was derived. Low stringency conditions are well known to those of skill in the art, 35 and will vary depending on the specific organisms from which the library and the labeled

sequence are derived. For guidance regarding such conditions see, for example, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Press, N.Y., and Ausabel *et al.*, 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y., each of which is incorporated herein by reference in its entirety. Further, mammalian OSN regulatory region homologues may be isolated from, for example, bovine or other non-human nucleic acid, by performing polymerase chain reaction (PCR) amplification using two primer pools designed on the basis of the nucleotide sequence of the OSN regulatory region disclosed herein. The template for the reaction may be cDNA obtained by reverse transcription of the mRNA prepared from, for example, bovine or other non-human cell lines, or tissue known to express the OSN gene. For guidance regarding such conditions, see, e.g., Innis *et al.* (Eds.) 1995, *PCR Strategies*, Academic Press Inc., San Diego; and Erlich (ed) 1992, *PCR Technology*, Oxford University Press, New York, each of which is incorporated herein by reference in its entirety.

Promoter sequences within the 5' non-coding regions of the OSN gene may be further defined by constructing nested 5' and/or 3' deletions using conventional techniques such as exonuclease III or appropriate restriction endonuclease digestion. The resulting deletion fragments can be inserted into the promoter reporter vector to determine whether the deletion has reduced or obliterated promoter activity, such as described, for example, by Coles *et al.* (Hum. Mol. Genet., 7:791-800, 1998). In this way, the boundaries of the promoters may be defined. If desired, potential individual regulatory sites within the promoter may be identified using site directed mutagenesis or linker scanning to obliterate potential transcription factor binding sites within the promoter individually or in combination. The effects of these mutations on transcription levels may be determined by inserting the mutations into cloning sites in promoter reporter vectors. These types of assays are well known to those skilled in the art (WO 97/17359, US 5,374,544, EP 582 796, US 5,698,389, US 5,643,746, US5,502,176, and US 5,266,488).

The OSN regulatory regions and transcriptionally functional fragments thereof, and the fragments and probes described herein which serve to identify OSN regulatory regions and fragments thereof, may be produced by recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct these sequences, either in isolated form or contained in expression vectors. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* genetic recombination. See, e.g., the techniques described in Sambrook *et al.*, 1989, *supra*, and Ausabel *et al.*, 1989, *supra*; also

see the techniques described in "Oligonucleotide Synthesis", 1984, Gait M.J. ed., IRL Press, Oxford, which is incorporated herein by reference in its entirety.

Alterations in the regulatory sequences can be generated using a variety of chemical and enzymatic methods which are well known to those skilled in the art. For
5 example, regions of the sequences defined by restriction sites can be deleted.

Oligonucleotide-directed mutagenesis can be employed to alter the sequence in a defined way and/or to introduce restriction sites in specific regions within the sequence.

Additionally, deletion mutants can be generated using DNA nucleases such as Bal31, ExoIII, or S1 nuclease. Progressively larger deletions in the regulatory sequences are
10 generated by incubating the DNA with nucleases for increased periods of time (see, e.g., Ausubel *et al.*, 1989, *supra*).

The altered sequences are evaluated for their ability to direct expression of heterologous coding sequences in appropriate host cells. It is within the scope of the present invention that any altered regulatory sequences which retain their ability to direct
15 expression of a coding sequence be incorporated into recombinant expression vectors for further use.

5.2 Analysis of Osteotropic-Specific Promoter Activity

The OSN regulatory region shows selective tissue and cell-type specificity;
20 i.e., it induces gene expression in osteotropic cells. Thus, the regulatory region, and transcriptionally active fragments thereof, of the present invention may be used to induce expression of a heterologous coding sequence specifically in osteotropic cells. The present invention provides for the use of the OSN regulatory region to achieve tissue specific expression of a target coding sequence. The activity and the specificity of the OSN
25 regulatory region can further be assessed by monitoring the expression level of a detectable polynucleotide operably associated with the OSN promoter in different types of cells, tissues and cell lines engineered to contain the OSN promoter. As discussed hereinbelow, the detectable polynucleotide may be either a polynucleotide that specifically hybridizes with a predefined oligonucleotide probe, or a polynucleotide encoding a detectable protein.

30

5.2.1 OSN Promoter Driven Reporter Constructs

The regulatory polynucleotides according to the invention may be advantageously part of a recombinant expression vector that may be used to express a coding sequence, or reporter gene, in a desired host cell or host organism. The OSN
35 regulatory region of the present invention, and transcriptionally active fragments thereof, may be used to direct the expression of a heterologous coding sequence. In particular, the

present invention encompasses mammalian OSN regulatory regions. In accordance with the present invention, transcriptionally active fragments of the OSN regulatory region encompass those fragments of the region which are of sufficient length to promote transcription of a reporter coding sequence to which the fragment is operatively linked.

5 A variety of reporter gene sequences well known to those of skill in the art can be utilized, including, but not limited to, genes encoding fluorescent proteins such as green fluorescent protein (GFP), enzymes (*e.g.* CAT, beta-galactosidase, luciferase) or antigenic markers. For convenience, enzymatic reporters and light-emitting reporters analyzed by colorometric or fluorometric assays are preferred for the screening assays of the
10 invention.

In one embodiment, for example, a bioluminescent, chemiluminescent or fluorescent protein can be used as a light-emitting reporter in the invention. Types of light-emitting reporters, which do not require substrates or cofactors, include, but are not limited to the wild-type green fluorescent protein (GFP) of *Victoria aequoria* (Chalfie *et al.*, 1994,
15 Science 263:802-805), and modified GFPs (Heim *et al.*, 1995, Nature 373:663-4; PCT publication WO 96/23810). Transcription and translation of this type of reporter gene leads to the accumulation of the fluorescent protein in test cells, which can be measured by a fluorimeter, or a flow cytometer, for example, by methods that are well known in the art (see, *e.g.*, Lackowicz, 1983, Principles of Fluorescence Spectroscopy, Plenum Press, New
20 York).

Another type of reporter gene that may be used are enzymes that require cofactor(s) to emit light, including but not limited to, Renilla luciferase. Other sources of luciferase also are well known in the art, including, but not limited to, the bacterial luciferase (*luxAB* gene product) of *Vibrio harveyi* (Karp, 1989, Biochim. Biophys. Acta
25 1007:84-90; Stewart *et al.* 1992, J. Gen. Microbiol, 138:1289-1300), and the luciferase from firefly, *Photinus pyralis* (De Wet *et al.* 1987, Mol. Cell. Biol. 7:725-737), which can be assayed by light production (Miyamoto *et al.*, 1987, J. Bacteriol. 169:247-253; Loessner *et al.* 1996, Environ. Microbiol. 62:1133-1140; and Schultz & Yarus, 1990, J. Bacteriol.
172:595-602).

30 Reporter genes that can be analyzed using colorimetric analysis include, but are not limited to, β -galactosidase (Nolan *et al.* 1988, Proc. Natl. Acad. Sci. USA 85:2603-07), β -glucuronidase (Roberts *et al.* 1989, Curr. Genet. 15:177-180), luciferase (Miyamoto *et al.*, 1987, J. Bacteriol. 169:247-253), or β -lactamase. In one embodiment, the reporter gene sequence comprises a nucleotide sequence which encodes a *LacZ* gene product, β -
35 galactosidase. The enzyme is very stable and has a broad specificity so as to allow the use of different histochemical, chromogenic or fluorogenic substrates, such as, but not limited

to, 5-bromo-4-chloro-3-indoyl- β -D-galactoside (X-gal), lactose 2,3,5-triphenyl-2H-tetrazolium (lactose-tetrazolium) and fluorescein galactopyranoside (see Nolan *et al.*, 1988, *supra*).

- In another embodiment, the product of the *E. coli* β -glucuronidase gene 5 (GUS) can be used as a reporter gene (Roberts *et al.* 1989, *Curr. Genet.* 15:177-180). GUS activity can be detected by various histochemical and fluorogenic substrates, such as X-glucuronide (Xgluc) and 4-methylumbelliferyl glucuronide.

In addition to reporter gene sequences such as those described above, which provide convenient colorimetric responses, other reporter gene sequences, such as, for 10 example, selectable reporter gene sequences, can routinely be employed. For example, the coding sequence for chloramphenicol acetyl transferase (CAT) can be utilized, leading to OSN regulatory region-dependent expression of chloramphenicol resistant cell growth. The use of CAT and the advantages of a selectable reporter gene are well known to those skilled in the art (Eikmanns *et al.* 1991, *Gene* 102:93-98). Other selectable reporter gene sequences 15 also can be utilized and include, but are not limited to, gene sequences encoding polypeptides which confer zeocin (Hegedus *et al.* 1998, *Gene* 207:241-249) or kanamycin resistance (Friedrich & Soriano, 1991, *Genes. Dev.* 5:1513-1523).

Other coding sequences, such as toxic gene products, potentially toxic gene products, and antiproliferation or cytostatic gene products, also can be used. In another 20 embodiment, the detectable reporter polynucleotide may be either a polynucleotide that specifically hybridizes with a predefined oligonucleotide probe, or a polynucleotide encoding a detectable protein, including an OSN polypeptide or a fragment or a variant thereof. This type of assay is well known to those skilled in the art (US 5,502,176 and US 5,266,488).

OSN promoter driven reporter constructs can be constructed according to 25 standard recombinant DNA techniques (see, e.g., *Methods in Enzymology*, 1987, volume 154, Academic Press; Sambrook *et al.* 1989, *Molecular Cloning - A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, New York; and Ausubel *et al.* *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, New York, each 30 of which is incorporated herein by reference in its entirety).

Methods for assaying promoter activity are well-known to those skilled in the art (see, e.g., Sambrook *et al.*, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). An example of a typical method that can be used involves a recombinant vector carrying a reporter gene and genomic sequences 35 from the OSN sequence depicted in Figure 1. Briefly, the expression of the reporter gene (for example, green fluorescent protein, luciferase, β -galactosidase or chloramphenicol

acetyl transferase) is detected when placed under the control of a biologically active polynucleotide fragment. Genomic sequences located upstream of the first exon of the gene may be cloned into any suitable promoter reporter vector. For example, a number of commercially available vectors can be engineered to insert the OSN regulatory region of the invention for expression in mammalian host cells. Non-limiting examples of such vectors are pSEAPBasic, pSEAP-Enhancer, p β gal-Basic, p β gal-Enhancer, or pEGFP-1 Promoter Reporter vectors (Clontech, Palo Alto, CA) or pGL2-basic or pGL3-basic promoterless luciferase reporter gene vector (Promega, Madison, WI). Each of these promoter reporter vectors include multiple cloning sites positioned upstream of a reporter gene encoding a readily assayable protein such as secreted alkaline phosphatase, green fluorescent protein, luciferase or β -galactosidase. The regulatory sequences of the OSN gene are inserted into the cloning sites upstream of the reporter gene in both orientations and introduced into an appropriate host cell. The level of reporter protein is assayed and compared to the level obtained with a vector lacking an insert in the cloning site. The presence of an elevated expression level in the vector containing the insert with respect the control vector indicates the presence of a promoter in the insert.

Expression vectors that comprise an OSN regulatory region may further contain a gene encoding a selectable marker. A number of selection systems may be used, including but not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026) and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:817) genes, which can be employed in tk⁻, hprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler *et al.*, 1980, Proc. Natl. Acad. Sci. USA 77:3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30:147) genes. Additional selectable genes include trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histidol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) and glutamine synthetase (Bebbington *et al.*, 1992, Biotech 10:169).

5.2.2 Characterization of Transcriptionally Active Regulatory Fragments

A fusion construct comprising an OSN regulatory region, or a fragment thereof, can be assayed for transcriptional activity. As a first step in promoter analysis, the transcriptional start point (+1 site) of the osteotropic-specific gene under study has to be determined using primer extension assay and/or RNAase protection assay, following standard methods (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, Cold Spring Harbor Press). The DNA sequence upstream of the +1 site is generally considered as the promoter region responsible for gene regulation. However, downstream sequences, including sequences within introns, also may be involved in gene regulation. To begin testing for promoter activity, a -3 kb to +3 kb region (where +1 is the transcriptional start point) may be cloned upstream of the reporter gene coding region. Two or more additional reporter gene constructs also may be made which contain 5' and/or 3' truncated versions of the regulatory region to aid in identification of the region responsible for osteotropic-specific expression. The choice of the type of reporter gene is made based on the application.

In a preferred embodiment, a GFP reporter gene construct is used. The application of green fluorescent protein (GFP) as a reporter is particularly useful in the study of osteotropic-specific gene promoters. A major advantage of using GFP as a reporter lies in the fact that GFP can be detected in freshly isolated tumor and tissue cells with calcification potential without the need for substrates.

In another embodiment of the invention, a *Lac Z* reporter construct is used. The *Lac Z* gene product, β -galactosidase, is extremely stable and has a broad specificity so as to allow the use of different histochemical, chromogenic or fluorogenic substrates, such as, but not limited to, 5-bromo-4-chloro-3-indoyl- β -D-galactoside (X-gal), lactose 2,3,5-triphenyl-2H-tetrazolium (lactose-tetrazolium) and fluorescein galactopyranoside (see Nolan *et al.*, 1988, *supra*).

For promoter analysis in transgenic mice, GFP that has been optimized for expression in mammalian cells is preferred. The promoterless cloning vector pEGFP1 (Clontech, Palo Alto, CA) encodes a red shifted variant of the wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells (Cormack *et al.*, 1996, Gene 173:33; Haas *et al.*, 1996, Curr. Biol. 6: 315). Moreover, since the maximal excitation peak of this enhanced GFP (EGFP) is at 488 nm, commonly used filter sets such as fluorescein isothiocyanate (FITC) optics which illuminate at 450-500 nm can be used to visualize GFP fluorescence. pEGFP1 proved to be useful as a reporter vector for promoter analysis in transgenic mice (Okabe *et al.*, 1997, FEBS Lett. 407: 313).

In an alternate embodiment, transgenic mice containing transgenes with an OSN regulatory region upstream of a luciferase reporter gene are utilized.

Putative promoter fragments can be prepared (usually from a parent phage clone containing 8-10 kb genomic DNA including the promoter region) for cloning using methods known in the art. In one embodiment, for example, promoter fragments are cloned into the multiple cloning site of a luciferase reporter vector. In one embodiment, restriction endonucleases are used to excise the regulatory region fragments to be inserted into the reporter vector. However, the feasibility of this method depends on the availability of proper restriction endonuclease sites in the regulatory fragment. In a preferred embodiment, the required promoter fragment is amplified by polymerase chain reaction (PCR; Saiki *et al.*, 1988, *Science* 239:487) using oligonucleotide primers bearing the appropriate sites for restriction endonuclease cleavage. The sequence necessary for restriction cleavage is included at the 5' end of the forward and reverse primers which flank the regulatory fragment to be amplified. After PCR amplification, the appropriate ends are generated by restriction digestion of the PCR product. The promoter fragments, generated by either method, are then ligated into the multiple cloning site of the reporter vector following standard cloning procedures (Sambrook *et al.*, 1989; *supra*). It is recommended that the DNA sequence of the PCR generated promoter fragments in the constructs be verified prior to generation of transgenic animals. The resulting reporter gene construct will contain the putative promoter fragment located upstream of the reporter gene open reading frame, e.g., GFP or luciferase cDNA.

In a preferred embodiment, the following protocol is used. Fifty to 100 pg of the reporter gene construct is digested using appropriate restriction endonucleases to release the transgene fragment. The restriction endonuclease cleaved products are resolved in a 1% (w/v) agarose gel containing 0.5 ug/ml ethidium bromide and TAE buffer (IX: 0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 5-6 V/cm. The transgene band is located by size using a UV transilluminator, preferably using long-wavelength UV lamp to reduce nicking of DNA, and the gel piece containing the required band carefully excised. The gel slice and 1 ml of 0.5 X TAE buffer is added to a dialysis bag, which has been boiled in 1 mM EDTA, pH 8.0 for 10 minutes (Sambrook *et al.*, 1989, *supra*) and the ends are fastened. The dialysis bag containing the gel piece is submerged in a horizontal gel electrophoresis chamber containing 0.5 X TAE buffer, and electrophoresed at 5-6 V/cm for 45 minutes. The current flow in the electrophoresis chamber is reversed for one minute before stopping the run to release the DNA which may be attached to the wall of the dialysis tube. The TAE buffer containing the electroeluted DNA from the dialysis bag is collected in a fresh eppendorf tube. The gel

piece may be observed on the UV transilluminator to ascertain that the electroelution of the DNA is complete.

The electroeluted DNA sample is further purified by passing through Elutip D columns. The matrix of the column is prewashed with 1-2 ml of High salt buffer (1.0 M NaCl, 20mM Tris. Cl, 1.0 mM EDTA, pH 7.5), followed by a wash with 5 ml of Low salt buffer (0.2 M NaCl, 20 mM Tris. Cl, 1.0 mM EDTA, pH 7.5). A 5 ml syringe is used to apply solutions to the Elutip D column, avoiding reverse flow. The solution containing the electroeluted DNA is loaded slowly. The column is washed with 2-3 ml of Low salt buffer and the DNA is eluted in 0.4 ml of High salt buffer. Two volumes of cold 95% ethanol is added to precipitate DNA. The DNA is collected by centrifugation in a microcentrifuge at 14,000 g for 10 minutes, carefully removing the alcohol without disrupting the DNA pellet. The pellet is washed at least twice with 70% (v/v) ethanol, and dried. The washing and drying steps are important, as residual salt and ethanol are lethal to the developing embryos. The DNA is resuspend in the injection buffer (10mM TM, 0.1 mM EDTA, pH 7.5 prepared with Milli-Q quality water). The concentration of the purified transgene DNA fragment is determined by measuring the optical density at A_{260} ($A_{260} = 1$ for 50 μ g/ml DNA) using a spectrophotometer. DNA prepared in this manner is suitable for microinjection into fertilized mouse eggs.

20 **5.2.3 Osteotropic-Specific Promoter Analysis Using Transgenic Mice**

The mammalian OSN regulatory region can be used to direct expression of, *inter alia*, a reporter coding sequence, a homologous gene or a heterologous gene in transgenic animals specifically within tumor and tissue cells with calcification potential. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, 25 micro-pigs, goats, sheep, and non-human primates, *e.g.*, baboons, monkeys and chimpanzees may be used to generate transgenic animals. The term "transgenic," as used herein, refers to non-human animals expressing OSN gene sequences from a different species (*e.g.*, mice expressing human OSN sequences), as well as animals that have been genetically engineered to over-express endogenous (*i.e.*, same species) OSN sequences or 30 animals that have been genetically engineered to knock-out specific sequences.

In one embodiment, the present invention provides for transgenic animals that carry a transgene such as a reporter gene, therapeutic and/or toxic coding sequence under the control of the OSN regulatory region, or transcriptionally active fragments thereof, in all their cells, as well as animals that carry the transgene in some, but not all their 35 cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also

be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (1992, Proc. Natl. Acad. Sci. USA 89:6232-6236). When it is desired that the transgene be integrated into the chromosomal site of the endogenous corresponding gene, gene targeting is preferred. Briefly, when such a technique 5 is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene.

Any technique known in the art may be used to introduce a transgene under 10 the control of the OSN regulatory region into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe & Wagner, 1989, U.S. Patent No. 4,873,191); nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal or adult cells induced to quiescence (Campbell *et al.*, 1996, Nature 380:64-66; Wilmut *et al.*, Nature 385:810-813); 15 retrovirus gene transfer into germ lines (Van der Putten *et al.*, 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, Cell 65:313-321); electroporation of embryos (Lo, 1983, Mol. Cell. Biol. 31:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, Cell 57:717-723; see, Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229).

20 For example, for microinjection of fertilized eggs, a linear DNA fragment (the transgene) containing the regulatory region, the reporter gene and the polyadenylation signals, is excised from the reporter gene construct. The transgene may be gel purified by methods known in the art, for example, by the electroelution method. Following 25 electroelution of gel fragments, any traces of impurities are further removed by passing through Elutip D column (Schleicher & Schuell, Dassel, Germany).

In a preferred embodiment, the purified transgene fragment is microinjected 30 into the male pronuclei of fertilized eggs obtained from B6 CBA females by standard methods (Hogan, 1986, Manipulating the Mouse Embryo, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Mice are analyzed transiently at several embryonic stages or by establishing founder lines that allow more detailed analysis 35 of transgene expression throughout development and in adult animals. Transgene presence is analyzed by PCR using genomic DNA purified from placentas (transients) or tail clips (founders) according to the method of Vemet *et al.*, *Methods Enzymol.* 1993;225:434-451. Preferably, the PCR reaction is carried out in a volume of 100 µl containing 1 µg of genomic DNA, in 1X reaction buffer supplemented with 0.2 mM dNTPs, 2 mM MgCl₂, 600 µM each of primer, and 2.5 units of *Taq* polymerase (Promega, Madison, WI). Each of the

30 PCR cycles consists of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min. The founder mice are then mated with C57B1 partners to generate transgenic F₁ lines of mice.

5 5.3 Screening Assays

Compounds that interfere with the abnormal function and/or growth of tumor and tissue cells with calcification potential can provide therapies targeting defects in osteotropic-related disorders including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, breast and prostate 10 cancers, and benign conditions, such as benign prostatic hyperplasia (BPH) or arterial sclerotic conditions where calcification occurs. Such compounds may be used to interfere with the onset or the progression of osteotropic-related disorders. Compounds that stimulate or inhibit promoter activity also may be used to ameliorate symptoms of osteotropic-related disorders.

15 Genetically engineered cells, cell lines and/or transgenic animals containing an OSN regulatory region, or fragment thereof, operably linked to a reporter gene, can be used as systems for the screening of agents that modulate OSN regulatory region activity. Such transgenic mice provide an experimental model *in vivo* (or can be used as a source of primary cells or cell lines for use *in vitro*) which can be used to develop new methods of 20 treating osteotropic-related disorders by targeting therapeutic and/or toxic agents to cause arrest in the progression of such disorders.

The present invention encompasses screening assays designed to identify compounds that modulate activity of the OSN regulatory region. The present invention encompasses *in vitro* and cell-based assays, as well as *in vivo* assays in transgenic animals. 25 As described hereinbelow, compounds to be tested may include, but are not limited to, oligonucleotides, peptides, proteins, small organic or inorganic compounds, antibodies, *etc.*

Examples of compounds may include, but are not limited to, peptides, such as, for example, soluble peptides, including, but not limited to, Ig-tailed fusion peptides, and members of random peptide libraries; (see, *e.g.*, Lam, *et al.*, 1991, *Nature* 354:82-84; 30 Houghten, *et al.*, 1991, *Nature* 354:84-86), and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang, *et al.*, 1993, *Cell* 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain

antibodies, and FAb, F(ab')₂ and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

Such compounds may further comprise compounds, in particular drugs or members of classes or families of drugs, known to ameliorate the symptoms of an
5 osteotropic-related disorder.

Such compounds include, but are not limited to, families of antidepressants such as lithium salts, carbamazepine, valproic acid, lysergic acid diethylamide (LSD), *p*-chlorophenylalanine, *p*-propyldopacetamide dithiocarbamate derivatives *e.g.*, FLA 63; anti-anxiety drugs, *e.g.*, diazepam; monoamine oxidase (MAO) inhibitors, *e.g.*, iproniazid,
10 clorgyline, phenelzine and isocarboxazid; biogenic amine uptake blockers, *e.g.*, tricyclic antidepressants such as desipramine, imipramine and amitriptyline; serotonin reuptake inhibitors *e.g.*, fluoxetine; antipsychotic drugs such as phenothiazine derivatives (*e.g.*, chlorpromazine (thorazine) and trifluoperazine)), butyrophenones (*e.g.*, haloperidol (Haldol)), thioxanthene derivatives (*e.g.*, chlorprothixene), and dibenzodiazepines (*e.g.*, clozapine); benzodiazepines; dopaminergic agonists and antagonists *e.g.*, L-DOPA, cocaine, amphetamine, α -methyl-tyrosine, reserpine, tetrabenazine, benzotropine, pargyline; noradrenergic agonists and antagonists *e.g.*, clonidine, phenoxybenzamine, phentolamine, tropolone; nitrovasodilators (*e.g.*, nitroglycerine, nitroprusside as well as NO synthase
15 enzymes); and growth factors (*e.g.*, VEGF, FGF, angiopoetins and endostatin).

In one preferred embodiment, genetically engineered cells, cell lines or primary cultures of germ and/or somatic cells containing a mammalian OSN regulatory region operatively linked to a heterologous gene are used to develop assay systems to screen for compounds which can inhibit sequence-specific DNA-protein interactions. Such
25 methods comprise contacting a compound to a cell that expresses a gene under the control of an OSN regulatory region, or a transcriptionally active fragment thereof, measuring the level of the gene expression or gene product activity and comparing this level to the level of gene expression or gene product activity produced by the cell in the absence of the compound, such that if the level obtained in the presence of the compound differs from that
30 obtained in its absence, a compound capable of modulating the expression of the mammalian OSN regulatory region has been identified. Alterations in gene expression levels may be by any number of methods known to those of skill in the art *e.g.*, by assaying for reporter gene activity, assaying cell lysates for mRNA transcripts, *e.g.* by Northern analysis or using other methods known in the art for assaying for gene products expressed
35 by the cell.

In another embodiment, microdissection and transillumination can be used. These techniques offer a rapid assay for monitoring effects of putative drugs on osteotropic cells in transgenic animals containing an OSN regulatory region-driven reporter gene. In this embodiment, a test agent is delivered to the transgenic animal by any of a variety of methods. Methods of introducing a test agent may include oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal and via scarification (scratching through the top layers of skin, e.g., using a bifurcated needle) or any other standard routes of drug delivery. The effect of such test compounds on the osteotropic cells can be analyzed by the microdissection and transillumination of the osteoblastic cells. If the level of reporter gene expression observed or measured in the presence of the compound differs from that obtained in its absence, a compound capable of modulating the expression of the mammalian OSN regulatory region has been identified.

In various embodiments of the invention, compounds that may be used in screens for modulators of osteotropic-related disorders include peptides, small molecules, both naturally occurring and/or synthetic (e.g., libraries of small molecules or peptides), cell-bound or soluble molecules, organic, non-protein molecules and recombinant molecules that may have OSN regulatory region binding capacity and, therefore, may be candidates for pharmaceutical agents.

Alternatively, the proteins and compounds include endogenous cellular components which interact with OSN regulatory region sequences *in vivo*. Cell lysates or tissue homogenates may be screened for proteins or other compounds which bind to the OSN regulatory region, or fragment thereof. Such endogenous components may provide new targets for pharmaceutical and therapeutic interventions.

In one embodiment, libraries can be screened. Many libraries are known in the art that can be used, e.g., peptide libraries, chemically synthesized libraries, recombinant (e.g., phage display libraries), and *in vitro* translation-based libraries. In one embodiment of the present invention, peptide libraries may be used to screen for agonists or antagonists of OSN-linked reporter expression. Diversity libraries, such as random or combinatorial peptide or non-peptide libraries can be screened for molecules that specifically modulate OSN regulatory region activity. Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to activate or inhibit OSN regulatory region activities (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that stimulate or inhibit the expression of OSN regulatory regions.

Examples of chemically synthesized libraries are described in Fodor *et al.*, 1991, Science 251:767-773; Houghten *et al.*, 1991, Nature 354:84-86; Lam *et al.*, 1991, Nature 354:82-84; Medynski, 1994, BioTechnology 12:709-710; Gallop *et al.*, 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten *et al.*, 1992, Biotechniques 13:412; Jayawickreme *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin *et al.*, 1990, Science, 249:404-406; Christian, *et al.*, 1992, J. Mol. Biol. 227:711-718; Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay *et al.*, 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

By way of example of non-peptide libraries, a benzodiazepine library (*see e.g.*, Bunin *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) also can be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh *et al.* (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

A specific embodiment of such an *in vitro* screening assay is described below. The OSN regulatory region-reporter vector is used to generate transgenic mice from which primary cultures of OSN regulatory region-reporter vector germ cells are established. About 10,000 cells per well are plated in 96-well plates in total volume of 100 μ l, using medium appropriate for the cell line. Candidate inhibitors of the OSN regulatory region are added to the cells. The effect of the inhibitors of the OSN regulatory region can be determined by measuring the response of the reporter gene driven by the OSN regulatory region. This assay could easily be set up in a high-throughput screening mode for evaluation of compound libraries in a 96-well format that reduce (or increase) reporter gene activity, but which are not cytotoxic. After 6 hours of incubation, 100 μ l DMEM medium + 2.5% fetal bovine serum (FBS) to 1.25% final serum concentration is added to the cells, which are incubated for a total of 24 hours (18 hours more). At 24 hours, the plates are washed with PBS, blot dried, and frozen at -80°C. The plates are thawed the next day and analyzed for the presence of reporter activity.

In a preferred example of an *in vivo* screening assay, tumor or tissue cells with calcification potential derived from transgenic mice can be transplanted into mice with a normal or other desired phenotype (Brinster *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91: 11298-302; Ogawa *et al.*, 1997, Int. J. Dev. Biol. 41:111-12). Such mice can then be used
5 to test the effect of compounds and other various factors on osteotropic-related disorders. In addition to the compounds and agents listed above, such mice can be used to assay factors or conditions that can be difficult to test using other methods, such as dietary effects, internal pH, temperature, *etc.*

Once a compound has been identified that inhibits or enhances OSN
10 regulatory region activity, it may then be tested in an animal-based assay to determine if the compound exhibits the ability to act as a drug to ameliorate and/or prevent symptoms of an osteotropic-related disorder, including, but not limited to, localized or disseminated osteosarcoma, lung, colon, melanoma, thyroid, brain, multiple myeloma, breast and prostate cancers, and benign conditions, such as benign prostatic hyperplasia (BPH) or arterial
15 sclerotic conditions where calcification occurs.

The assays of the present invention may be first optimized on a small scale (*i.e.*, in test tubes), and then scaled up for high-throughput assays. The screening assays of the present invention may be performed *in vitro*, *i.e.*, in test tubes, using purified components or cell lysates. The screening assays of the present invention may also be
20 carried out in intact cells in culture and in animal models. In accordance with the present invention, test compounds which are shown to modulate the activity of the OSN regulatory region *in vitro*, as described herein, will further be assayed *in vivo* in cultured cells and animal models to determine if the test compound has the similar effects *in vivo* and to determine the effects of the test compound on osteotropic-related disorders.
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5.4 Compositions and Methods for Therapeutic Use of OSN Regulatory Region Nucleotides

OSN regulatory regions, or transcriptionally active fragments thereof, can be used to treat and/or prevent diseases, conditions or disorders that can be ameliorated by
30 modifying the level or the expression of OSN, or a heterologous gene linked to an OSN regulatory region, in an osteotropic-specific manner. Described herein are methods for such therapeutic treatments.

The OSN regulatory region may be used to achieve tissue specific expression in gene therapy protocols. In cases where such cells are tumor cells, the induction of a
35 cytotoxic product by the OSN regulatory region may be used in the form of cancer gene therapy specifically targeted to tumor cells with calcification potential which contain trans-

acting factors required for OSN expression. In this way, the OSN regulatory region may serve as a delivery route for a gene therapy approach to cancers involving tumor cells with calcification potential. Additionally, antisense, antigene or aptameric oligonucleotides may be delivered to cells using the presently described expression constructs. Ribozymes or 5 single-stranded RNA also can be expressed in a cell to inhibit the expression of a target gene of interest. The target genes for these antisense or ribozyme molecules should be those encoding gene products that are essential for cell maintenance.

The OSN regulatory region, and transcriptionally active fragments thereof, of the present invention may be used for a wide variety of purposes, e.g., to down regulate 10 OSN gene expression, or, alternatively, to achieve osteotropic-specific expression of heterologous coding sequences.

In one embodiment, for example, the endogenous OSN regulatory region may be targeted to specifically down-regulate expression of the OSN gene. For example, oligonucleotides complementary to the regulatory region may be designed and delivered to 15 the cells. Such oligonucleotides may anneal to the regulatory sequence and prevent transcription activation. Alternatively, the regulatory sequence, or portions thereof, may be delivered to cells in saturating concentrations to compete for transcription factor binding. For general reviews of the methods of gene therapy, see Goldspiel *et al.*, 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. 20 Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11:155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory 25 Manual, Stockton Press, NY.

In another embodiment, a gene therapy method for ameliorating osteotropic-related disorders is provided. OSN regulatory region sequences are introduced in the osteotropic cells and used to drive osteotropic-specific expression of drugs or toxins. The method comprises introducing an OSN regulatory region sequence operatively associated 30 with a drug or toxin gene into the osteotropic cells.

In yet another embodiment, the invention provides a gene therapy method for treatment of cancer or other proliferative disorders. The OSN regulatory region is used to direct the expression of one or more proteins specifically in osteotropic tumor cells of a patient. Such proteins may be, for example, tumor suppressor genes, thymidine kinase 35 (used in combination with acyclovir), toxins or proteins involved in cell killing, such as proteins involved in the apoptosis pathway.

In one embodiment, the invention provides for a therapeutic agent comprising an OSN promoter which is useful for toxic gene therapy. This method includes a eukaryotic delivery vector and a toxic gene. In the preferred embodiment, the vector is adenovirus (Ad) and the gene is thymidine kinase (TK). Thus, the therapeutic agent is 5 represented by the formula Ad-OSN-TK, but in reality the novel concept contained herein is the OSN promoter as the driving force for osteotropic-specific expression of heterologous coding sequences.

The DNA encoding the translational or transcriptional products of interest may be engineered recombinantly into a variety of vector systems that provide for 10 replication of the DNA in large scale for the preparation of the vectors of the invention. These vectors can be designed to contain the necessary elements for directing the transcription and/or translation of the DNA sequence taken up by the osteotropic cells.

Vectors that may be used include, but are not limited to, those derived from recombinant bacteriophage DNA, plasmid DNA or cosmid DNA. For example, plasmid 15 vectors such as pBR322, pUC 19/18, pUC 118, 119 and the M13 mp series of vectors may be used. Bacteriophage vectors may include λgt10, λgt11, λgt18-23, λZAP/R and the EMBL series of bacteriophage vectors. Cosmid vectors that may be utilized include, but are not limited to, pJB8, pCV 103, pCV 107, pCV 108, pTM, pMCS, pNNL, pHSG274, COS202, COS203, pWE15, pWE16 and the charomid 9 series of vectors. Vectors that 20 allow for the *in vitro* transcription of RNA, such as SP6 vectors, also may be used to produce large quantities of RNA that may be incorporated into viral vectors.

Alternatively, recombinant replication competent or incompetent viral vectors including, but not limited to, those derived from viruses such as herpes virus, retroviruses, vaccinia viruses, adenoviruses, adeno-associated viruses or bovine papilloma 25 virus may be engineered. While integrating vectors may be used, non-integrating systems, which do not transmit the gene product to daughter cells for many generations, are preferred for non-disease related repair and regeneration. In this way, the gene product is expressed during the repair process, and as the gene is diluted out in progeny generations, the amount of expressed gene product is diminished.

30 The use of tissue specific promoters to drive therapeutic gene expression would decrease further a toxic effect of the therapeutic gene on neighboring normal cells when virus-mediated gene delivery results in the infection of the normal cells. This would be important especially in diseases where systemic administration could be utilized to deliver a therapeutic vector throughout the body, while maintaining transgene expression to 35 a limited and specific number of cell types. Moreover, since many bone growth factors,

such as TGF- β , have pleiotropic effects, numerous, harmful side effects likely would be exhibited if the growth factor genes are expressed in all cells.

In some instances, the promoter elements may be constitutive or inducible promoters and can be used under the appropriate conditions to direct high level or regulated expression of the gene of interest. Expression of genes under the control of constitutive promoters does not require the presence of a specific substrate to induce gene expression and will occur under all conditions of cell growth. In contrast, expression of genes controlled by inducible promoters is responsive to the presence or absence of an inducing agent. For example, if a cell is stably transfected with a therapeutic, inducible transgene, its expression could be controlled over the life-time of the individual. In fact, the OSN promoter, itself, is induced by glucocorticoids and ascorbic acid.

Specific initiation signals also are required for sufficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire coding sequence, including the initiation codon and adjacent sequences, are inserted into the appropriate expression vectors, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the protein coding sequences to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency and control of expression may be enhanced by the inclusion of transcription attenuation sequences, enhancer elements, etc.

In another embodiment of the present invention there is provided a method for treating osteotropic tumors comprising delivering a therapeutic agent to the tumor. The therapeutic agent comprises a recombinant adenovirus vector (Ad) containing an OSN promoter driven toxic thymidine kinase (Tk). An additional aspect of the present invention provides a method of regulating expression of Tk with the addition of a suitable prodrug including, but not limited to, acyclovir (AcV).

The therapeutic agent containing the OSN promoter-driven toxic gene therapy, in the presence of a suitable prodrug, can be administered to osteosarcoma tumors, and prostate cancer tumors, and their metastases, and many other osteotropic tumors, including, but not limited to, colon, brain, lung, breast, multiple, myeloma, thyroid, and melanoma. The present therapeutic invention comprising Ad-OSN-TK, or other vectors containing OSN promoter-driven activity, is provided to target cancers that are osteotropic,

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thus possessing bone-like and bone homing capabilities and eliciting an osteoblastic or osteolytic phenotype when in association with bone tissue.

In still another embodiment, the OSN regulatory region may code for a variety of factors that promote bone repair including extracellular, cell surface and intracellular RNAs and proteins. These therapeutic constructs would be useful for, *inter alia*, aging and certain degenerative conditions. Examples of extracellular proteins include growth factors, cytokines, therapeutic proteins, hormones and peptide fragments of hormones, inhibitors of cytokines, peptide growth and differentiation factors, interleukins, chemokines, interferons, colony stimulating factors and angiogenic factors. Examples of such proteins include, but are not limited to, the superfamily of TGF- β molecules, including the five TGF- β isoforms and bone morphogenetic proteins (BMP), latent TGF- β binding proteins, LTBP; keratinocyte growth factor (KGF); hepatocyte growth factor (HGF); platelet derived growth factor (PDGF); insulin-like growth factor (IGF); the basic fibroblast growth factors (FGF-1, FGF-2, etc.), vascular endothelial growth factor (VEGF); Factor VIII and Factor IX; erythropoietin (EPO); tissue plasminogen activator (TPA) and activins and inhibins. Hormones which may be used in the practice of the invention include, for example, growth hormone (GH) and parathyroid hormone (PTH). Examples of extracellular proteins also include the extracellular matrix proteins such as collagen, laminin and fibronectin. Examples of cell surface proteins include the family of cell adhesion molecules (e.g., the integrins, selectins, Ig family members such as N-CAM and L1 and cadherins); cytokine signaling receptors such as the type I and type II TGF- β receptors and the FGF receptor and non-signaling co-receptors such as betaglycan and syndecan. Examples of intracellular RNAs and proteins include the family of signal transducing kinases, cytoskeletal proteins such as talin and vinculin, cytokine binding proteins such as the family of latent TGF- β binding proteins and nuclear *trans* acting proteins such as transcription factors and enhancing factors.

The method comprises introducing an OSN regulatory region sequence operatively associated with a nucleic acid encoding a therapeutic compound that promotes bone synthesis and/or repair. The tissue specificity of the OSN promoter will allow for specific expression of the therapeutic compounds in osteotropic cells of interest. The use of the OSN promoter to drive therapeutic gene expression would decrease further a toxic effect of the therapeutic gene on neighboring normal cells when virus-mediated gene delivery results in the infection of the normal cells. This would be important especially in diseases where systemic administration could be utilized to deliver a therapeutic vector throughout the body, while maintaining transgene expression to a limited and specific number of cell

types. Moreover, since many therapeutic growth factors, such as TGF- β , have pleiotropic effects, numerous, harmful side effects likely would be exhibited if the growth factor genes are expressed in all cells.

In yet another embodiment, the OSN regulatory region may code for a variety 5 of genes with immune modulatory functions, e.g. for cytokines such as interleukins 1 to 15 inclusive, especially for example IL2, IL12, gamma-interferon, tumour necrosis factor, GMCSF, and/or other genes, e.g. those mentioned in specifications WO 88/00971 (CSIRO, Australian National University: Ramshaw et al) and WO 94/16716 (Virogenetics Corp; Paoletti et al).

10 Also the following genes can be encoded by the OSN regulatory regions of the invention: genes for interferons alpha, beta or gamma; tumour necrosis factor; granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (N-CSF), chemokines such as neutrophil activating protein NAP, macrophage chemoattractant and 15 activating factor MCAF, RANTES, macrophage inflammatory peptides MIP-1a and MIP-1b, complement components and their receptors, accessory molecules such as 87.1, 87.2, ICAM-1.2 or 3 or cytokine receptors. Where nucleotide sequences encoding more than one immunomodulating protein are inserted, they may comprise more than one cytokine or may represent a combination of cytokine and accessory molecule(s).

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5.4.1 Modulatory Antisense, Ribozyme and Triple Helix Approaches

In another embodiment, the types of conditions, disorders, or diseases involving tumor and tissue cells with calcification potential which may be prevented, delayed, or rescued by modulating osteotropic-specific gene expression by using an OSN 25 regulatory region in conjunction with well-known antisense, gene "knock-out," ribozyme and/or triple helix methods, are described. Such molecules may be designed to modulate, reduce or inhibit either unimpaired, or if appropriate, mutant osteotropic gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

30 Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense approaches involve the design of oligonucleotides which are complementary to an mRNA sequence. The antisense oligonucleotides will bind to the complementary mRNA sequence transcripts and prevent translation. Absolute complementarity, although preferred, is not 35 required.

A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The 5 ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

10 In one embodiment, oligonucleotides complementary to non-coding regions of the sequence of interest could be used in an antisense approach to inhibit translation of endogenous mRNA. Antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at 15 least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit sequence expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. 20 Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleic acid of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

25 The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in* 30 *vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger, *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre, *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, *e.g.*, Krol *et al.*, 1988, *BioTechniques* 35 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide,

hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

- The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil,
- 5 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine,
- 10 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-
- 15 3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

- In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

- In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier, *et al.*, 1987, *Nucl. Acids Res.* 15:6625-6641). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue, *et al.*, 1987, *Nucl. Acids Res.* 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue, *et al.*, 1987, *FEBS Lett.* 215:327-330).

- Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein, *et al.* (1988, *Nucl. Acids Res.* 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin, *et al.*, 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:7448-7451), etc.

While antisense nucleotides complementary to the osteotropic specific coding region sequence could be used, those complementary to the transcribed, untranslated region are most preferred.

Antisense molecules should be delivered to cells that express the osteotropic sequence *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies which specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

A preferred approach to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs which will form complementary base pairs with the endogenous sequence transcripts and thereby prevent translation of the mRNA. For example, a vector can be introduced *e.g.*, such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3'-long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, 1982, *Nature* 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct that can be introduced directly into the tissue site. Alternatively, viral vectors can be used that selectively infect the desired tissue, in which case administration may be accomplished by another route (*e.g.*, systemically).

Ribozyme molecules designed to catalytically cleave target gene mRNA transcripts can also be used to prevent translation of target gene mRNA and, therefore, expression of target gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver, *et al.*, 1990, *Science* 247, 1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, *Current Biology* 4:469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event.

- 5 The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, e.g., U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences 10 can be used to destroy target gene mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions which form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in 15 Myers, 1995, *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and Gerlach, 1988, *Nature*, 334:585-591, which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is 20 located near the 5' end of the target gene mRNA, i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been extensively 25 described by Thomas Cech and collaborators (Zaug, et al., 1984, *Science*, 224:574-578; Zaug and Cech, 1986, *Science*, 231:470-475; Zaug, et al., 1986, *Nature*, 324:429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, *Cell*, 47:207-216). The Cech-type ribozymes have an eight base pair active site that hybridizes to a target RNA sequence whereafter cleavage of the target RNA 30 takes place. The invention encompasses those Cech-type ribozymes that target eight base-pair active site sequences that are present in the target gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g., for improved stability, targeting, etc.) and should be delivered to cells that express the target gene *in vivo*. A preferred method of delivery involves using a DNA 35 construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to

destroy endogenous target gene messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous target gene expression can also be reduced by inactivating or
5 "knocking out" the target gene or its promoter using targeted homologous recombination
(e.g., see Smithies, *et al.*, 1985, *Nature* 317:230-234; Thomas and Capecchi, 1987, *Cell*
51:503-512; Thompson, *et al.*, 1989, *Cell* 5:313-321; each of which is incorporated by
reference herein in its entirety). For example, a mutant, non-functional target gene (or a
completely unrelated DNA sequence) flanked by DNA homologous to the endogenous
10 target gene (either the coding regions or regulatory regions of the target gene) can be used,
with or without a selectable marker and/or a negative selectable marker, to transfect cells
which express the target gene *in vivo*. Insertion of the DNA construct, via targeted
homologous recombination, results in inactivation of the target gene. Such approaches are
particularly suited in the agricultural field where modifications to ES (embryonic stem) cells
15 can be used to generate animal offspring with an inactive target gene (e.g., see Thomas and
Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for
use in humans provided the recombinant DNA constructs are directly administered or
targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, endogenous target gene expression can be reduced by targeting
20 deoxyribonucleotide sequences complementary to the regulatory region of the target gene
(*i.e.*, the target gene promoter and/or enhancers) to form triple helical structures which
prevent transcription of the target gene in target cells in the body. (See generally, Helene,
1991, *Anticancer Drug Des.*, 6(6):569-584; Helene, *et al.*, 1992, *Ann. N.Y. Acad. Sci.*,
660:27-36; and Maher, 1992, *Bioassays* 14(12):807-815).

25 Nucleic acid molecules to be used in triple helix formation for the inhibition
of transcription should be single stranded and composed of deoxynucleotides. The base
composition of these oligonucleotides must be designed to promote triple helix formation
via Hoogsteen base pairing rules, which generally require sizable stretches of either purines
or pyrimidines to be present on one strand of a duplex. Nucleic acids may be pyrimidine-
30 based, which will result in TAT and CGC⁺ triplets across the three associated strands of the
resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a
purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In
addition, nucleic acid molecules may be chosen which are purine-rich, for example, contain
a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is
35 rich in GC pairs, in which the majority of the purine residues are located on a single strand
of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a 5 sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, and/or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal target gene alleles which 10 the possibility may arise wherein the concentration of normal target gene product present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of target gene activity are maintained, therefore, nucleic acid molecules which encode and express target gene polypeptides exhibiting normal target gene activity may be introduced into cells via gene therapy methods such as those described, 15 below, in Section 5.4.2 which do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the target gene encodes an extracellular protein, it may be preferable to co-administer normal target gene protein in order to maintain the requisite level of target gene activity.

Anti-sense RNA and DNA, ribozyme, and triple helix molecules of the 20 invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid-phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the 25 antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

30

5.4.2 Gene Replacement Therapy

The nucleic acid sequences of the invention, described above, can be utilized for transferring recombinant nucleic acid sequences to cells and expressing said sequences in recipient cells. Such techniques can be used, for example, in marking cells or for the 35 treatment of a disorder involving tumor or tissue cells with calcification potential. Such treatment can be in the form of gene replacement therapy. Specifically, one or more copies

of a normal gene or a portion of the gene that directs the production of a gene product exhibiting normal gene function, may be inserted into the appropriate cells within a patient, using vectors that include, but are not limited to adenovirus, adeno-associated virus, herpes simplex virus and retrovirus vectors, in addition to other particles that introduce DNA into 5 cells, such as liposomes.

Methods for introducing genes for expression in mammalian cells are well known in the field. Generally, for such gene therapy methods, the nucleic acid is directly administered *in vivo* into a target cell or a transgenic mouse that expresses an OSN regulatory region operably linked to a heterologous coding sequencee. This can be 10 accomplished by any method known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), by direct injection of naked DNA, by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), by coating with lipids or cell-surface 15 receptors or transfecting agents, by encapsulation in liposomes, microparticles, or microcapsules, by administering it in linkage to a peptide which is known to enter the nucleus or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), which can be used to target cell types specifically expressing the receptors. In another embodiment, a nucleic 20 acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April 16, 1992; WO 92/22635 dated December 23, 1992; WO92/20316 dated November 26, 25 1992; WO93/14188 dated July 22, 1993; WO 93/20221 dated October 14, 1993). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438).

In one embodiment, techniques for delivery involve direct administration, 30 *e.g.*, by stereotactic delivery of such gene sequences to the site of the cells in which the gene sequences are to be expressed.

Additional methods that may be utilized to increase the overall level of gene expression and/or gene product activity include using targeted homologous recombination methods, as discussed above, to modify the expression characteristics of an endogenous 35 gene in a cell or microorganism by inserting a heterologous DNA regulatory element such

that the inserted regulatory element is operatively linked with the endogenous gene in question. Targeted homologous recombination can thus be used to activate transcription of an endogenous gene that is "transcriptionally silent", *i.e.*, is not normally expressed or is normally expressed at very low levels, or to enhance the expression of an endogenous gene
5 that is normally expressed.

Further, the overall level of target gene expression and/or gene product activity may be increased by the introduction of appropriate target gene-expressing cells, preferably autologous cells, into a patient at positions and in numbers that are sufficient to ameliorate the symptoms of an osteotropic-related disorder. Such cells may be either
10 recombinant or non-recombinant.

When the cells to be administered are non-autologous cells, they can be administered using well known techniques that prevent a host immune response against the introduced cells from developing. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the
15 immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.
15

Additionally, compounds, such as those identified via techniques such as those described above that are capable of modulating activity of an OSN regulatory region can be administered using standard techniques that are well known to those of skill in the
20 art.

5.5 Pharmaceutical Preparations and Methods of Administration

The compounds that are determined to modify OSN regulatory region
25 activity or gene product activity can be administered to a patient at therapeutically effective doses to treat or ameliorate a disorder involving tumor or tissue cells with calcification potential. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of such a disorder.

30 5.5.1 Effective Dose

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and
35 therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit large therapeutic indices are preferred. While compounds that

exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used
5 in formulating a range of dosage for use in humans. The dosage of such compounds lies
preferably within a range of circulating concentrations that include the ED₅₀ with little or no
toxicity. The dosage may vary within this range depending upon the dosage form employed
and the route of administration utilized. For any compound used in the method of the
invention, the therapeutically effective dose can be estimated initially from cell culture
10 assays. A dose may be formulated in animal models to achieve a circulating plasma
concentration range that includes the IC₅₀ (*i.e.*, the concentration of the test compound that
achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such
information can be used to more accurately determine useful doses in humans. Levels in
plasma may be measured, for example, by high performance liquid chromatography.

15

5.5.2 Formulations and Use

Pharmaceutical compositions for use in accordance with the present
invention may be formulated in conventional manner using one or more physiologically
acceptable carriers or excipients.

20 Thus, the compounds and their physiologically acceptable salts and solvates
may be formulated for administration by inhalation or insufflation (either through the mouth
or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form
of, for example, tablets or capsules prepared by conventional means with pharmaceutically
25 acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch,
polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose,
microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium
stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or
wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well
30 known in the art. Liquid preparations for oral administration may take the form of, for
example, solutions, syrups or suspensions, or they may be presented as a dry product for
constitution with water or other suitable vehicle before use. Such liquid preparations may
be prepared by conventional means with pharmaceutically acceptable additives such as
suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats);
35 emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily

esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give
5 controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation
10 from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the
15 compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions
20 in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as
25 cocoa butter or other glycerides.

In certain embodiments, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment. This may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by
30 means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

For topical application, the compounds may be combined with a carrier so that an effective dosage is delivered, based on the desired activity.

- In addition to the formulations described previously, the compounds also may be formulated as a depot preparation. Such long acting formulations may be
- 5 administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.
- 10 The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

15 **6 EXAMPLE: Construction of OSN Promoter Deletion Constructs**

6.1 Materials and Methods

6.1.1 Cells and Cell Culture

LNCaP, C4-2, C4-2B, PC3, PC3M, P69, DU145, MG, MG63, 9069E, ROS and WH cells were maintained in T medium supplemented with 5% FBS. For transfection, 20 cells were growth in phenol-red free serum-free RPMI 1640 (Gibco BRL, MD). 293 cell line was purchased from Microbix Biosystems Inc. (Toronto, Ontario, Canada) and maintained in Minimal Eagles Medium (MEM) supplemented with 10% fetal calf serum and glutamine (Gibco BRL).

25 **6.1.2 Construction of OSN Promoter/Luciferase Constructs**

All promoter constructs were generated by TOPO TA cloning system (Invitrogen, CA) and subsequently digested using appropriate restriction sites in the polylinker to allow insertion into the vector pGL3-basic (Promega) containing the coding region of the firefly luciferase gene. The OSN 2.3, OSN 1.5, OSN 1.1 and OSN 0.2 promoter constructs were prepared using the primer sequences P1, P2, P3 and P4 as shown 30 in Figure 1. Additional OSN promoter deletion constructs starting at -522 were prepared. These constructs, which also contain a deletion of a spacer bewteen GGA box 1 and 2, contain -522 to +39, -522 to +62 and -522 to +73 of the OSN sequence (See Figures 1 and 11). To create these constructs beginning at -522 of the OSN sequence, PCR was performed 35 utilizing the following primers:

522-N: (5'ACTAGTAGCAGCTTGTCTTGTC3'), spdel-C:

(5'CTTCTCCCCGTCTCTGTCTT3'); and spdel-N:
(5'AAGACAGAGACAGGGGAGAAG3') combined with downstream primers: Intron-C:
(5'TACCTCAGTGGCAGGCAGGCAG3'), Exon-C:
(5'CAGGCAGGCAGGCCAG'), and Hafner-C:
5 (5'GCGCGCTCTCCGGGCAGTCTG3') to construct hON-522I, hON-522E and hON-Hafner, respectively, and the genomic DNA isolated from DU145 cells as template. All constructs, including the PCR-generated DNA fragments, were confirmed by sequencing.

6.2 Results

10 Four primer sets designated as P1, P2, P3, and P4 (see Figure 1 for sequence and position of the primers) were prepared. Figure 1 represents an OSN regulatory region from -1409 to +904. Also shown on Figure 1 are the GGA box-1, GGA box-2 and the boundary between exon 1 and intron 1. Similarly, Figure 11 represents a human osteonectin promoter sequence from -522 to +62 lacking a spacer between GGA box 1 and 2. Genomic
15 DNA extracted from a human prostate cancer cell line, DU-145, was used as a template to conduct the PCR reactions. The PCR products were subcloned and sequenced. The P1 and P4 primers generated a 2.3 kb OSN promoter fragment (OSN-2.3). The P2 and P4 primers generated a 1.1 kb promoter fragment (OSN-1.1), whereas P2 and P3 generated a 200 bp promoter fragment (OSN-0.2). Finally, the P3 and P4 primers generated a 1.5 kb promoter
20 fragment (OSN-1.5). These PCR products were sequenced and their identities were confirmed. Figure 2 is a schematic representation of the strategy used to construct the OSN promoter-mediated luciferase plasmids. Figure 7 is a schematic representation of the OSN sequence which was used to generate the various deletion constructs.

25 7 Example: OSN Promoter Activity

7.1 Materials and Methods

7.1.1 Transient DNA Transfection

ROS, LNCaP, C4-2 and C4-2B cells were seeded at 3.3×10^5 cells/well in 6-well plates 2 days before transfection. PC3, PC3M, DU145, MG63, 9069E and WH cells
30 were plated at 2×10^5 cells/well in 6-well plates 1 day prior to transfection. Plasmid DNA was introduced into cells by DOTAP reagent following the supplier's protocol (Boehringer Mannheim, IN). Briefly, the medium was replaced by 1 ml of serum-free and phenol-red free RPMI 1640 medium, and the cells were incubated for 6 h with a DOTAP-DNA mixture containing 3 μ g DNA and 10 μ l of DOTAP. The medium was then replaced by normal
35 growth medium. As an internal control for transfection efficiency, the cells were cotransfected in a 5:1 molar ratio with the vector pCMV- β -gal encoding the reporter gene

β -galactosidase.

7.1.2 Luciferase and β -galactosidase Assays

Cells were harvested 48 h after transfection and resuspended in 300 μ l of 1X lysis buffer according to the luciferase reporter gene system purchased from Promega (Madison, USA). Cell lysates were vortexed for a few seconds and centrifuged for 3 minutes. For the luciferase activity assay, 20 μ l of supernatant was mixed with 100 μ l of luciferase substrate (Promega, WI) and luciferase activity was measured by a luminometer (Monolight 2010, Analytical luminescence Laboratory, MD). For the β -gal activity assay, 100 μ l of supernatant was mixed with an equal volume of 2X β -gal substrate (Promega, WI) and then incubated at 37°C for 15-30 minutes. The β -gal activity was determined by a microplate reader at 405 nm wavelength. Luciferase activity was calculated relative to the β -galactosidase activity. As a reference control, the basic promoter vector pGL3-TATA was used. At least three independent transfections were performed in duplicate for each construct.

7.2 Results

The activities of the OSN-promoter deletion constructs were analyzed in both prostatic (LNCaP, C4-2, C4-2B, PC-3, P69) and non-prostatic (WH, MG) human epithelial cell lines with osteoblastic MG (MG-63) serving as a positive control (Figure 3). In addition, the activity of a human OC promoter (3.9 kb)-mediated luciferase construct was used as a positive control for confirming relative luciferase activity. Figure 3 shows that OSN-1.5 expressed the highest activity, as compared to OSN-0.2, OSN-1.1 and OSN-2.3. The high luciferase activity mediated by the OSN-1.5 promoter appeared to correlate with the steady-state level of OSN protein expression analyzed by western blot (Figure 4). These results extended a previous observation made by Hafner et al., Matrix Bio. 14:733, 1995, in which cell-specific activity was not contained in GGA box 1 (see Figure 1 for location of GGA boxes in OSN promoter). Figure 8 shows that hON522E had the highest RLA (relative luiferase activity) as compare with hONHafner and hON522I in various cancer cell lines including WH, PC3 and MG63. Figure 9 further shows a comparison of hON522E activity in different prostate cell lines, with PC3M cells having the highest activity.

A novel aspect of the present invention is that the expression of OSN in a cell type-specific manner was observed upon the removal of intron 1 and by extending the OSN promoter from GGA box 1 to include the entire exon 1 (Figure 1). These data demonstrate that inhibitory cis-elements that exist in the OSN promoter (e.g. intron 1) can

be removed to enhance OSN promoter activity and specificity that can be used to confer tissue specificity and be applied to cancer and bone diseases for therapeutic gains.

8 Example: Determination of OSN mRNA in Various Cell Lines and Prostate Tumor Tissues.

8.1 Materials and Methods

8.1.1 RT-PCR Analysis of Human Osteonectin Expression

RNA was extracted from cell lines and prostate tumor tissue using RNAzol B reagent (TEL-Test, INC, TX) following the manufacturer's instruction. One microgram of total RNA was used to synthesize the first-strand cDNA using random primers (Perkin Elmer) and MMLV reverse transcriptase (Gibco BRL) in a total volume of 20 μ l. The reaction was performed at 42°C for 1 hour. 2.5 μ l of the RT reaction was adjusted to contain 25 ng of each pair of human osteonectin-specific primers (5'TCCACCACCCTGTTGCTGT3', sense; and 5'CTCCAGGCGCTCTCATT3', antisense) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (5' ACCACAGTCCATGCCATCA3', sense; 5'TCCACCACCCTGTTGCTGT3', antisense); an additional buffer was added to a total volume of 25 μ l. PCR was then performed for 50 cycles (94°C, 30 second; 55°C, 30 second; and 72°C, 1 min) for detecting the expression of osteonectin and 25 cycles for detecting the expression of GAPDH as an internal control. Finally, the amplified products were separated on a 1.5% agarose gel.

8.2 Results

Figure 5 shows OSN expression in various prostate cancer cell lines, including LNCaP, C4-2, C4-2B, DU145, PC3, PC3M and MG63. GAPDH expression was also monitored as an internal control. Figure 6, shows OSN expression in primary prostate cancer tissues. Samples that revealed detectable OSN mRNA are in lanes 1, 2 and 5-7. Again, as with Figure 5, GAPDH expression was monitored as an internal control.

9 Example: *In Vitro* Cytotoxicity Assays with Ad-522E-TK

30 9.1 Materials and Methods

9.1.1 Construction and Production of Recombinant Ad-522E-TK

The Ad-522E-TK adenovirus (type 5) was constructed and mass-produced according to the standard protocol. Briefly, the plasmid p522E-TK containing a human osteonectin promoter and herpes simplex virus TK gene was constructed by inserting the expression cassette into the E1A deleted region of Ad5 adenoviral shuttle vector, pΔ Elsp1B. A replication-defective recombinant Ad522E-TK adenovirus was generated in 293

cells by co-transfected these cells with both the expression shuttle plasmid and a circular Ad genome plasmid (pJMI7) using the standard calcium-phosphate precipitation method. Briefly, low passage (<40) 293 cells were seeded in 60-mm dishes to be about 70-80% confluent at the time of use. 20 μ g shuttle plasmid DNA and 40 μ l Ad genome plasmid
5 were mixed in 2 ml HEBS (21 mM HEPES, 0.137 M NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 5.5 mM glucose, pH 7.1) containing 10 μ l of 2 mg/ml salmon sperm carrier DNA by tipping the tube, then slowly adding 100 μ l of 2.5 M CaCl₂ dropwise with gentle mixing. DNA precipitate were allowed to form for 15-30 minutes at room temperature. 0.5 ml of the DNA suspension was then added to each dish of cells without removing the growth
10 medium. After overnight culture, the growth medium was replaced by a 10 ml overlay (MEM+0.5% agarose). When the agarose solidified, cells were incubated at 37°C for up to 10-12 days to allow the formation of plaques. Ad-522E-TK virus was obtained by plaque purification according to the method of Graham and Prevec (Molecular Biotechnology 3: 207, 1995). Plaques were picked up and isolated from transfected cultures and infected 293
15 cells. Cells were harvested 36-48 hrs after infection, pelleted, resuspended in PBS, and lysed. The virus in the cell lysate was purified by CsCl₂ gradient centrifugation. After dialysis, the concentrated virus was evaluated by particle count as determined by optical density measurement of DNA, and stored at -80°C before experimentation.

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9.1.2 Ad-522E-TK plus GCV-induced In Citro Cytotoxicity

PC3M and MG63 cells were seeded onto 24-well plates at a density of 2x 10⁴ cells/well. After 24 hr, the cells were infected with PBS or increasing m.o.i. of Ad-522E-TK in 1 ml of growth medium. After overnight infection, the virus was removed and cells were incubated in fresh medium containing 0 or 10 μ g/ml of GCV for an additional 5 days.
25 Duplicate plates were fixed and stained with crystal violet.

9.2 Results

Figure 10A shows that PC3M cells with virus plus GCV did not proliferate as well as the cells without GCV. Similarly, Figure 10B shows the results using the MG-63
30 cell line. Similar to the results with PC3M, the virus plus GCV treated cells did not proliferate as much as the cells in the control group (without GCV).

10 Example: Inhibition of Tumor Growth *In Vivo***10.1 Materials and Methods****10.1.1 In Vivo Analysis of Recombinant Ad-522E-TK**

Male athymic nu/nu mice (Harlan Co., TX), 5-6 weeks of age, were inoculated subcutaneously with 5×10^5 PC3M cells in 50 μ l PBS into both sites of the flank. When the tumor became palpable (3-4 mm in diameter), the animals were randomly assigned to 4 experimental groups: group 1, PBS treatment; group 2, GCV only; group 3, Ad-522E-TK plus PBS; and group 4, Ad-522E-TK plus GCV. Fifty μ l of Ad-522E-TK (2×10^9 pfu) in PBS-10% glycerol was intratumorally injected on days 8, 11 and 14. One hundred μ l of GCV was administrated via intraperitoneal injection at a dose of 40 mg/kg body weight daily for 2 weeks. Bidimensional tumor measurements were performed at least once a week with calipers, and tumor volume was calculated using the simplified formula of a rotational ellipsoid ($l \times w^2 \times 0.5236$). Ko, S.C. et al., *Hum. Gene Ther.*, 7: 1683-1691, 1996.

15

10.2 Results

The data shown in Figure 12 demonstrate that the virus plus prodrug treated group had minimal tumor growth over the 21 day period of the experiment while the three control groups had quite a large increase in tumor growth. Thus, the data demonstrate that the Ad-522E-TK viral vector is effective at ameliorating and/or treating tumors with calcification potential *in vivo*.

10.3 Discussion

The present invention unexpectedly demonstrates that OSN promoter-driven genes, when infected into susceptible osteotropic tumors and cells of osteoblastic lineage, such as osteosarcomas (ROS, MG63, Saos-2), prostate (LNCaP, C4-2, C4-2B4 PC-3 PC3M, Du-145, ARCaP), colon (Lovo), lung (A547), brain (U-87), and breast (MCF-7) express very high levels of reporter gene, and thus are expected also to efficiently express high levels of chosen toxic or therapeutic gene when in combination with known delivery vector, including, but not limited to, Ad-OSN-TK. Further, the invention demonstrates that addition of a suitable prodrug, such as GCV, when combined with Ad-OSN-TK system, will effect the cessation, inhibition and cytotoxicity of said osteotropic tumors and their associated osteoblastic supporting stroma. Since OSN is primarily expressed in fully differentiated osteoblasts or tissues or tumors with the capability to mineralize, and this OSN promoter can drive efficient expression of chosen genes, either reporter, therapeutic or toxic, in osteotropic tumors as evidenced herein, the present invention unexpectedly teaches

that OSN promoter constructs constitute an essential tumor-specific gene therapy that can inhibit the growth of certain osteotropic tumors (osteosarcoma, prostate, etc.) while sparing the surrounding normal tissues, or non-osteoblastic or non-osteolytic lineage cells of significant damage, and when applied systemically can prevent destruction of inappropriate tissue, while still obtaining the desired destructive effect on tumor growth. Further, the OSN promoter-driven therapeutic gene therapy is superior to conventional gene therapies for osteotropic tumors when the therapeutic gene expression is driven by universal promoters such as cytomegalovirus (CMV) and the long terminal repeat promoters of Rous Sarcoma Virus (RSV), because these universal promoters cannot distinguish the specific targeted tumor, and thus may cause inappropriate damage to nonselected tissues by expression of toxic gene in normal cells.

As mentioned previously, one therapeutic application of OSN promoter-driven gene therapy is to target cancers having the ability to metastasize to the skeleton. One mechanism for malignant cell recruitment to bone is that the osteotroblastic cells may synthesize and secrete products that are able to stimulate the growth, adhesion, and migration of osteotropic tumors such as prostate and breast cancer, and thus the reciprocal relationship between foreign tumor epithelium (cancer) and supporting bone stroma provides a favorable environment (soil) for accelerated tumor growth. The proliferation and migration of prostate or breast tumor cells may also themselves secrete paracrine growth factors that stimulate osteoblast or osteoclast bone cell growth at sites of bone metastases that results in induction of predominantly osteoblastic (e.g. prostate) or osteolytic (e.g. breast) phenotypes in the skeleton of afflicted patients. Since tumor cell growth is intimately affected by the surrounding stroma and these reciprocal interactions exist between certain tumors (prostate, osteosarcoma, breast, etc.) and bone stroma (osteoblast, or osteoclast), the development of OSN promoter-based gene therapy to target tumors, their metastasis and the supporting stroma represents a new and highly specific gene therapy modality for patients afflicted with these tumors. Indeed, OSN expression is distinct even from the other truly osteoblast specific gene, osteocalcin. Thus, OSN driven toxic gene therapy, most commonly in the form of Ad-OSN-TK, exhibits various therapeutic implications: a) OSN promoter driven gene therapy (Ad-OSN-TK) affects the expression of toxic compounds to osteosarcoma and prostate cancer cells and also eradicates osteoblastic cells that may be required to maintain the survival of osseous metastatic deposits of certain osteotropic tumors. Moreover, although the therapy may eradicate the growth of some normal osteoblastic cells, this is not a concern since the expression of OSN is highly specific and should not unduly harm the host by expression of toxic compounds in a wide variety of tissues; b) OSN promoter driven gene application can express high levels of

therapeutic target genes in many calcified tumors, and is a reasonable choice for eradicating these tumors primary focus and its metastatic deposits in conjunction with the supporting stroma; c) OSN promoter-driven therapeutic gene treatments in conjunction with appropriate vehicles may be used in conjunction with conventional chemotherapy, surgery 5 or radiation techniques or other novel therapies in reducing tumor burden and associated morbidity in local and metastatic deposits associated with various susceptible osteotropic human or eukaryotic tumors; d) Long lasting anti-tumor immunity might be elected against the remaining osteoblastic cells and tumor cells from OSN promoter-driven killing of tumor cells; e) OSN-promoter driven constructs may be used for the delivery and expression of 10 therapeutic genes for the treatment of benign diseases such as BPH, arterial sclerosis, etc., and also the expression of crucial growth and differentiation associated genes such as growth factors, growth factor receptors, bone morphogenic proteins. etc. for repairing the damages acquired during aging and degenerative conditions; and f) OSN promoter driven therapy can be modulated by a variety of compounds to produce an enhanced desired effect 15 for the specific application intended.

Prostate cancer metastasizes primarily to the skeleton. The present invention demonstrates that OSN is expressed highly in a wide variety of prostate cancer cells, and that the OSN promoter can efficiently drive expression of chosen reporter gene and thus also drive a toxic and/or therapeutic gene when in conjunction with this promoter, and a 20 gene delivery vehicle. This expression includes both osteoblastic prostate cancer cells and osteolytic prostate cancer cells. Similarly, the present invention will also be useful in other tumors, including, but not limited to, melanoma, thyroid, gastric, ovarian, osteosarcoma, colon, lung, breast, and brain tumors.

Thus, a novel therapeutic agent comprising an OSN promoter linked with an 25 appropriate delivery vector and therapeutic (or toxic) gene is conceived to be generated, including, but not limited to, Ad-OSN-TK. This recombinant, novel system will efficiently express therapeutic action and selectively target and induce the killing of osteoblast lineage cells and a wide spectrum of tumors or other susceptible benign tissues that have acquired the potential ability to calcify.

30 Further, a new recombinant therapy agent, such as, but not limited to Ad-OSN-TK, will be available to patients afflicted not only with osteosarcoma or prostate cancer, but also lung, breast, thyroid, myeloma, melanoma, colon, brain, gastric, ovarian and other calcifying tumors, or susceptible benign tissues.

Other toxins or therapeutic genes may be used with the OSN promoter- 35 driven therapy instead of the mentioned Tk. These include genes for cytosine deaminase, tumor suppresser genes, cyclic regulatory proteins, including various cytokines, growth or

differentiation factors and others all can be ligated to OSN promoter in place of the mentioned Tk gene. In addition, other vector delivery systems can be generated and combined with the OSN promoter to effectively elicit the desired therapeutic response.

The invention described and claimed herein is not to be limited in scope by
5 the specific embodiments herein disclosed since these embodiments are intended as
illustration of several aspects of the invention. Any equivalent embodiments are intended to
be within the scope of this invention. Indeed, various modifications of the invention in
addition to those shown and described herein will become apparent to those skilled in the art
from the foregoing description. Such modifications are also intended to fall within the
10 scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

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WHAT IS CLAIMED IS:

1. A therapeutic agent comprising an OSN promoter, a delivery vector and a toxic, therapeutic and/or heterologous coding sequence.

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2. The therapeutic agent of claim 1, further comprising a prodrug.

3. The therapeutic agent of claim 2, wherein said prodrug is selected from the group consisting of acyclovir ("ACV") and gancyclovir ("GCV").

10

4. The therapeutic agent of claim 1, wherein said OSN promoter comprises nucleotides -1409 to +73 depicted in Figure 1.

15

5. The therapeutic agent of claim 1, wherein said OSN promoter comprises nucleotides -522 to +62 depicted in Figure 11.

6. The therapeutic agent of claim 1, wherein said delivery vector comprises a viral vector.

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7. The therapeutic agent of claim 6, wherein said viral vector is an adenovirus.

8. The therapeutic agent of claim 1, wherein said delivery vector comprises a liposome.

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9. The therapeutic agent of claim 1, wherein said toxic coding sequence is selected from the group consisting of thymidine kinase and cytosine deaminase.

10. The therapeutic agent of claim 1, wherein said therapeutic coding sequence is selected from the group consisting of growth factors, cytokines, therapeutic proteins, hormones and peptide fragments of hormones, inhibitors of cytokines, peptide growth and differentiation factors, interleukins, chemokines, interferons, colony stimulating factors and angiogenic factors.

11. The therapeutic agent of claim 1, wherein said heterologous coding sequence 35 is a reporter gene.

12. The therapeutic agent of claim 11, wherein said reporter gene is a luciferase.
13. A method for identifying a test compound capable of modulating osteotropic-specific gene expression comprising:
 - 5 (a) measuring the level of expression of a reporter gene under the control of an OSN regulatory region, or a transcriptionally active fragment thereof, in the presence and absence of said test compound, such that if the level obtained in the presence of the test compound differs from that obtained in its absence, then a compound which modulates osteotropic-specific gene expression is identified.
14. The method of claim 13 wherein the reporter gene is luciferase.
15. A pharmaceutical composition comprising the test compound identified by 15 the method in claim 13.
16. A method for delivery of a toxic and/or therapeutic molecule comprising, introducing into osteotropic cells of a subject a vector comprising an OSN regulatory region sequence, or transcriptionally active fragment thereof, operatively linked to a heterologous nucleic acid which encodes said toxic and/or therapeutic molecule.
17. A method for treating and/or ameliorating an osteotropic-related disease or disorder comprising introducing into osteotropic cells of a subject a vector comprising an OSN regulatory region sequence, or transcriptionally active fragment thereof, operatively 25 linked to a heterologous nucleic acid whose gene product is capable of treating and/or ameliorating said disease or disorder.
18. A method for treating and/or ameliorating an osteotropic-related cancer or other proliferative disorder comprising introducing into a cell of said cancer or other 30 proliferative disorder of a subject a vector comprising an OSN regulatory region sequence, or transcriptionally active fragment thereof, a delivery vector and a toxic, therapeutic and/or heterologous coding sequence whose gene product is capable of killing said cell.
19. The method of claim 18 wherein said cancer or other proliferative disorder

is selected from the group consisting of osteosarcoma, prostate, breast, colon, lung, brain, multiple myeloma, gastric, ovarian, thyroid, melanoma or any other disease or disorder with calcification potential.

5 20. The method of claim 18 further comprising introducing a prodrug.

21. The method of claim 20 wherein said prodrug is selected from the group consisting of ACV and GCV.

10 22. The method of claim 20 wherein said introducing comprises administration via direct application, or systemic application via intravenous administration, intra-arterial administration, intra-tumoral administration, perfusion and oral administration.

23. The method of claim 18, wherein said OSN promoter comprises nucleotides
15 -1409 to +73 depicted in Figure 1.

24. The method of claim 18, wherein said OSN promoter comprises nucleotides
-522 to +62 depicted in Figure 11.

20 25. The method of claim 23, wherein said OSN regulatory region sequence
comprises a nucleotide sequence which hybridizes under highly stringent conditions to the
complement of nucleotides -1409 to +73 depicted in Figure 1.

26. The method of claim 24, wherein said OSN regulatory region sequence
25 comprises a nucleotide sequence which hybridizes under highly stringent conditions to the
complement of nucleotides -522 to +62 depicted in Figure 11.

27. A method for preventing or delaying an osteotropic-related disorder
comprising introducing into osteotropic cells of a subject a vector comprising an OSN
30 regulatory region sequence, or transcriptionally active fragment thereof, operatively linked
to a heterologous nucleic acid which encodes a therapeutic molecule which is capable of
preventing or delaying said disorder.

28. A method for promoting bone repair comprising administering a
35 polynucleotide to an area where bone repair is necessary, wherein said polynucleotide

comprises an OSN regulatory region sequence, or transcriptionally active fragment thereof, a delivery vector and therapeutic coding sequence whose gene product is capable of promoting said bone repair.

5 29. The method of claim 28, wherein said therapeutic coding sequence is selected from the group consisting of growth factors, cytokines, therapeutic proteins, hormones and peptide fragments of hormones, inhibitors of cytokines, peptide growth and differentiation factors, interleukins, chemokines, interferons, colony stimulating factors and angiogenic factors.

10

30. A method for modulating immune functions comprising administering a polynucleotide to an area where modulation of immune function is necessary, wherein said polynucleotide comprises an OSN regulatory region sequence, or transcriptionally active fragment thereof, a delivery vector and therapeutic coding sequence whose gene product is 15 capable of modulating immune functions.

31. The method of claim 30, wherein said therapeutic coding sequence is selected from the group consisting of interferons alpha, beta or gamma; tumour necrosis factor; granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte 20 colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), chemokines such as neutrophil activating protein NAP, macrophage chemoattractant and activating factor MCAF, RANTES, macrophage inflammatory peptides MIP-1a and MIP-1b, complement components and their receptors, accessory molecules such as 87.1, 87.2, ICAM-1.2 or 3 or cytokine receptors.

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1 / 12

FIGURE 1

FIGURE 2

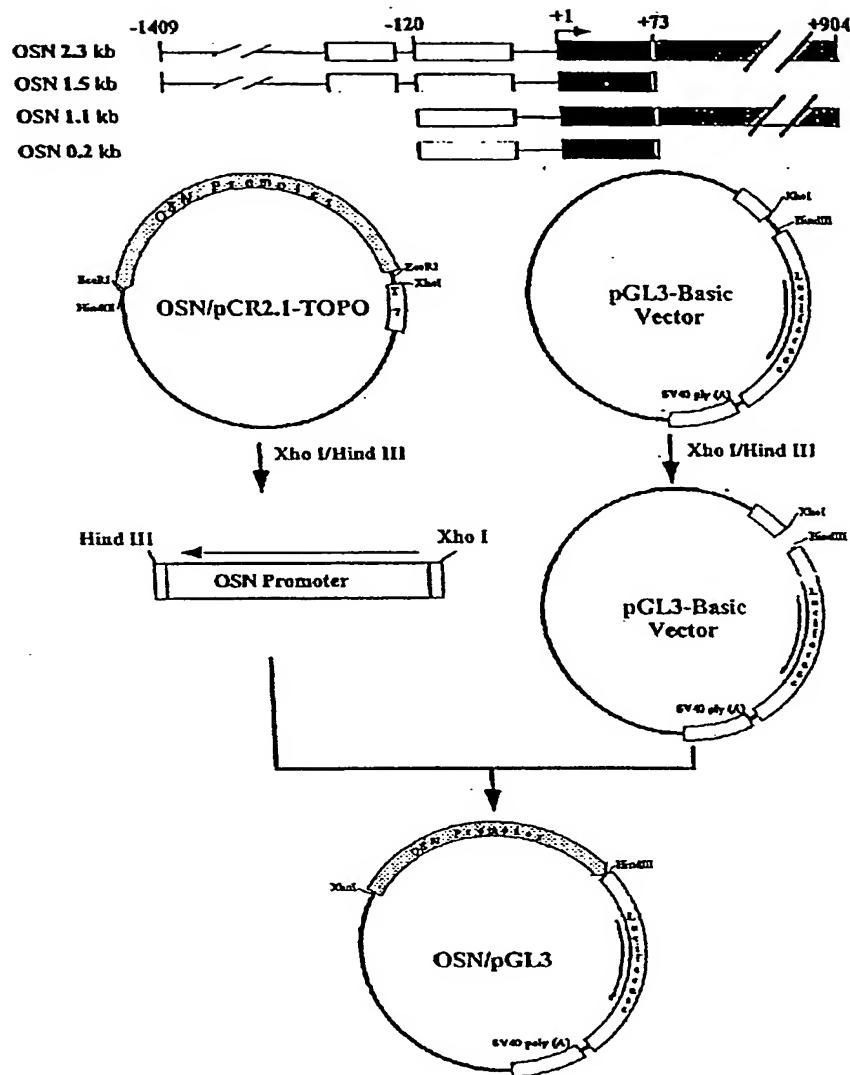


FIGURE 3

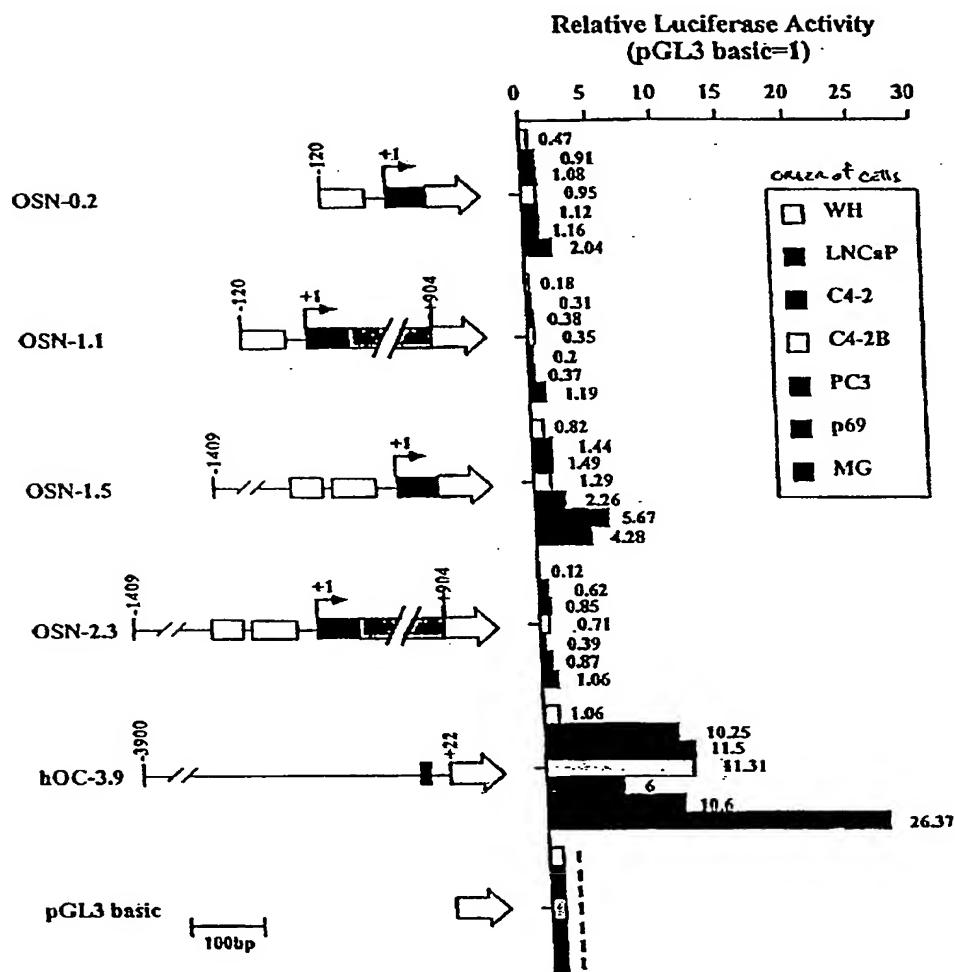
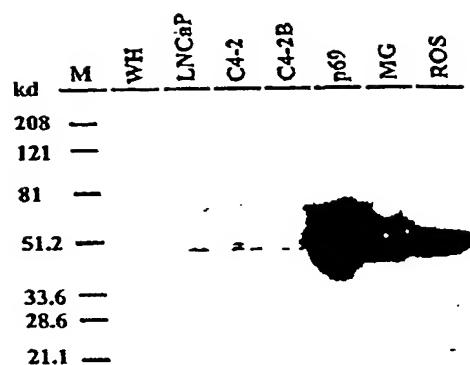


FIGURE 4



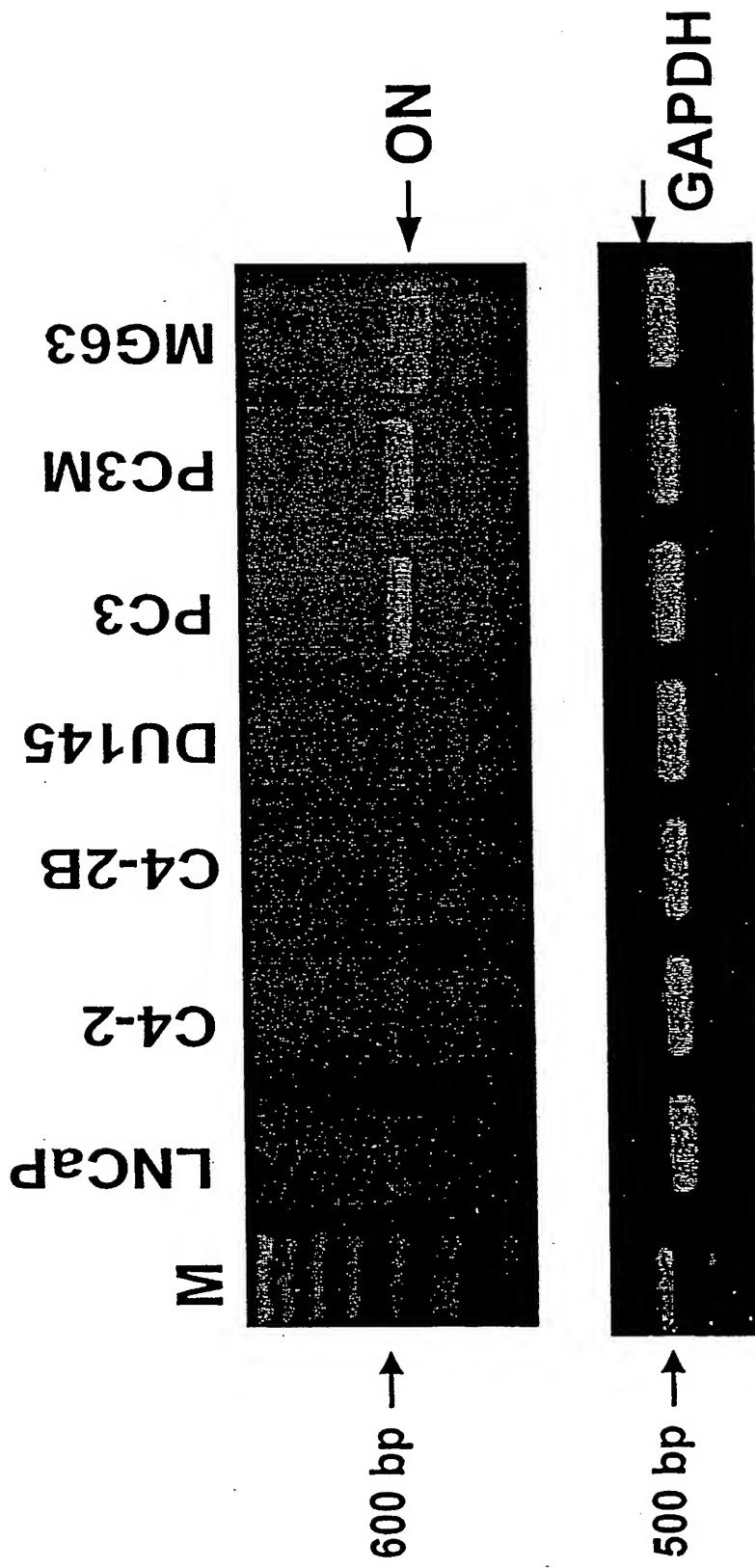


FIG. 5 Differential expression of osteonectin mRNA in prostate cancer cell lines. RNA from different prostate cancer cell lines and osteosarcoma cell line (MG63) was extracted and analyzed by RT-PCR. M, DNA marker.

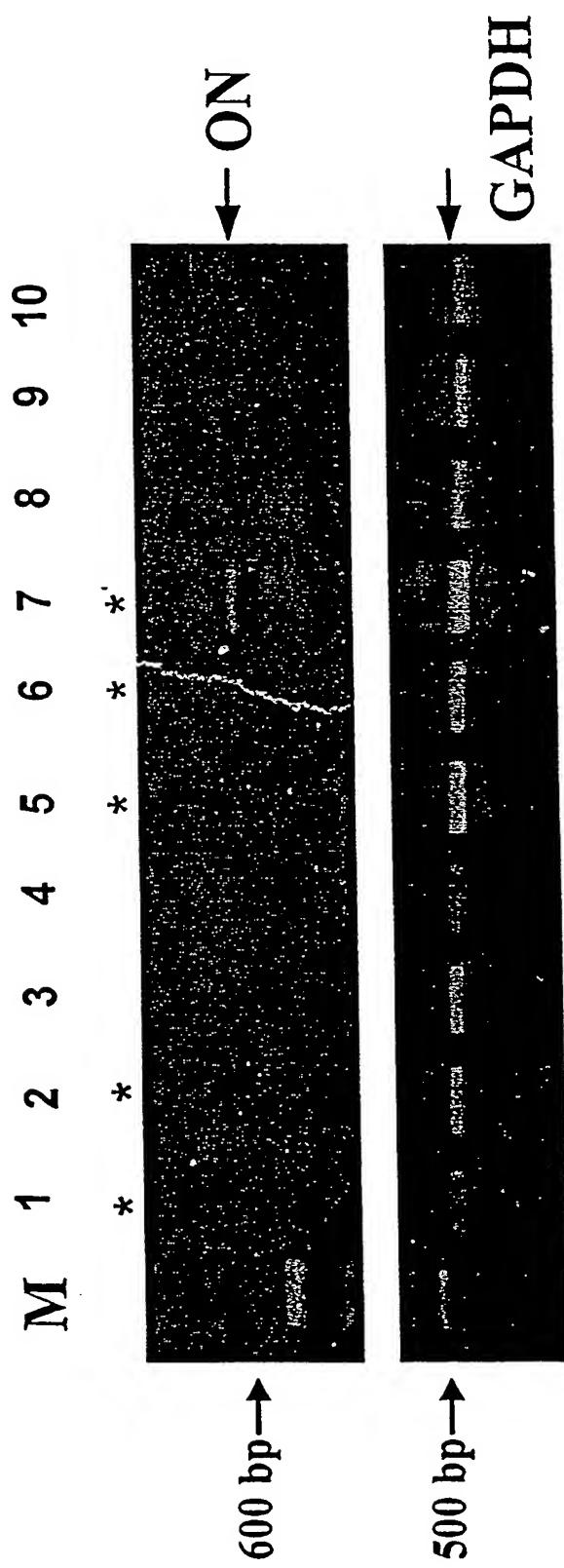


FIG. 6 Expression of osteonectin mRNA in prostate cancer tissues.
RNA from primary prostate cancer tissues was extracted and analyzed by RT-PCR. *, sample revealed detectable PCR product of ON. M, DNA marker.

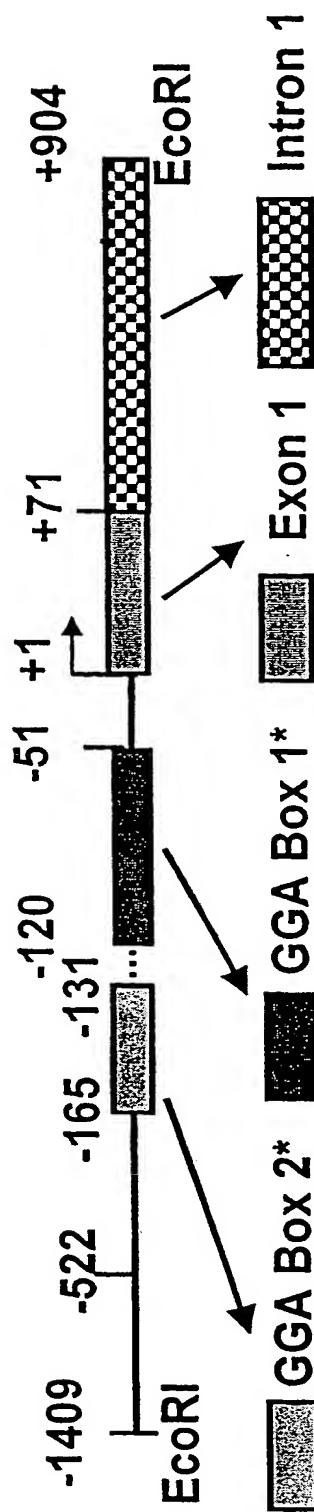


FIG. 7 Organization of human osteonectin gene promoter showing a 2.3 kb of EcoRI fragment, defined by M. Hafner *et al.*, 1994. * , enhancer; dash line, repressor; +1, transcriptional start site.

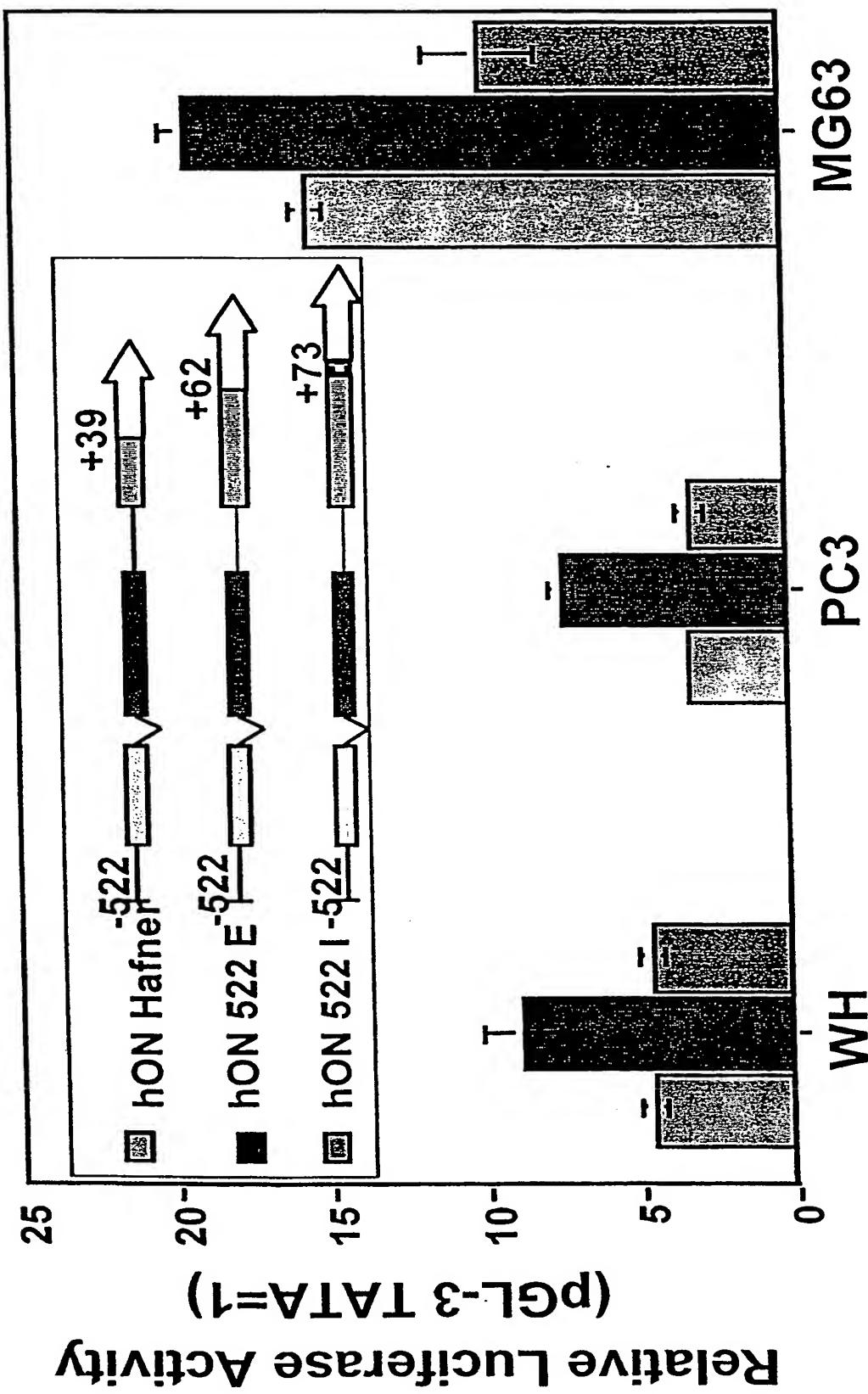


FIG. 8 Comparison of promoter activity among 3'-deleted hON promoter constructs in different cancer cell lines by luciferase activity assay.

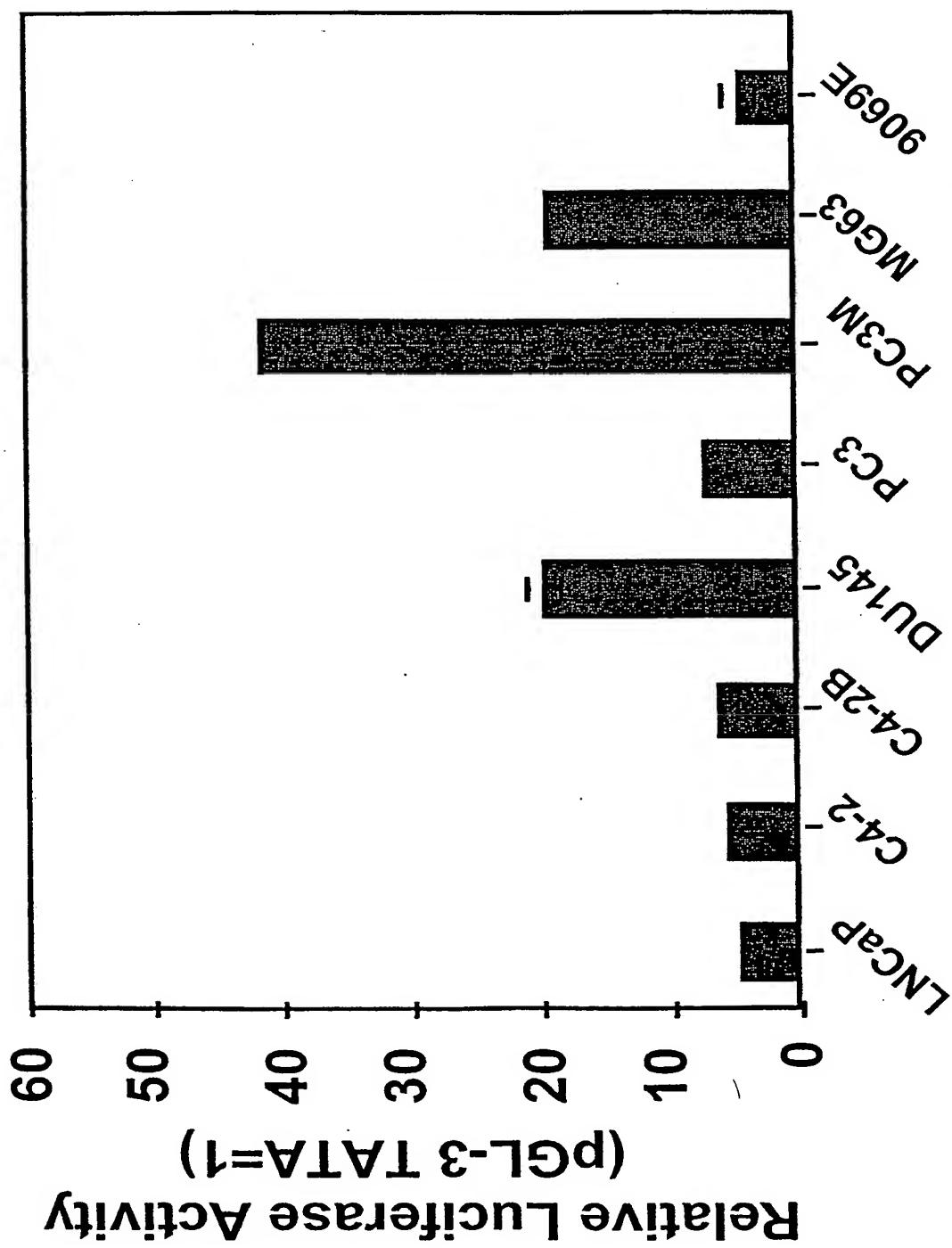


FIG. 9 Comparison of hON-522E promoter activity in different prostate cell lines by luciferase activity assay. 9069E, human prostate epithelium cell line.

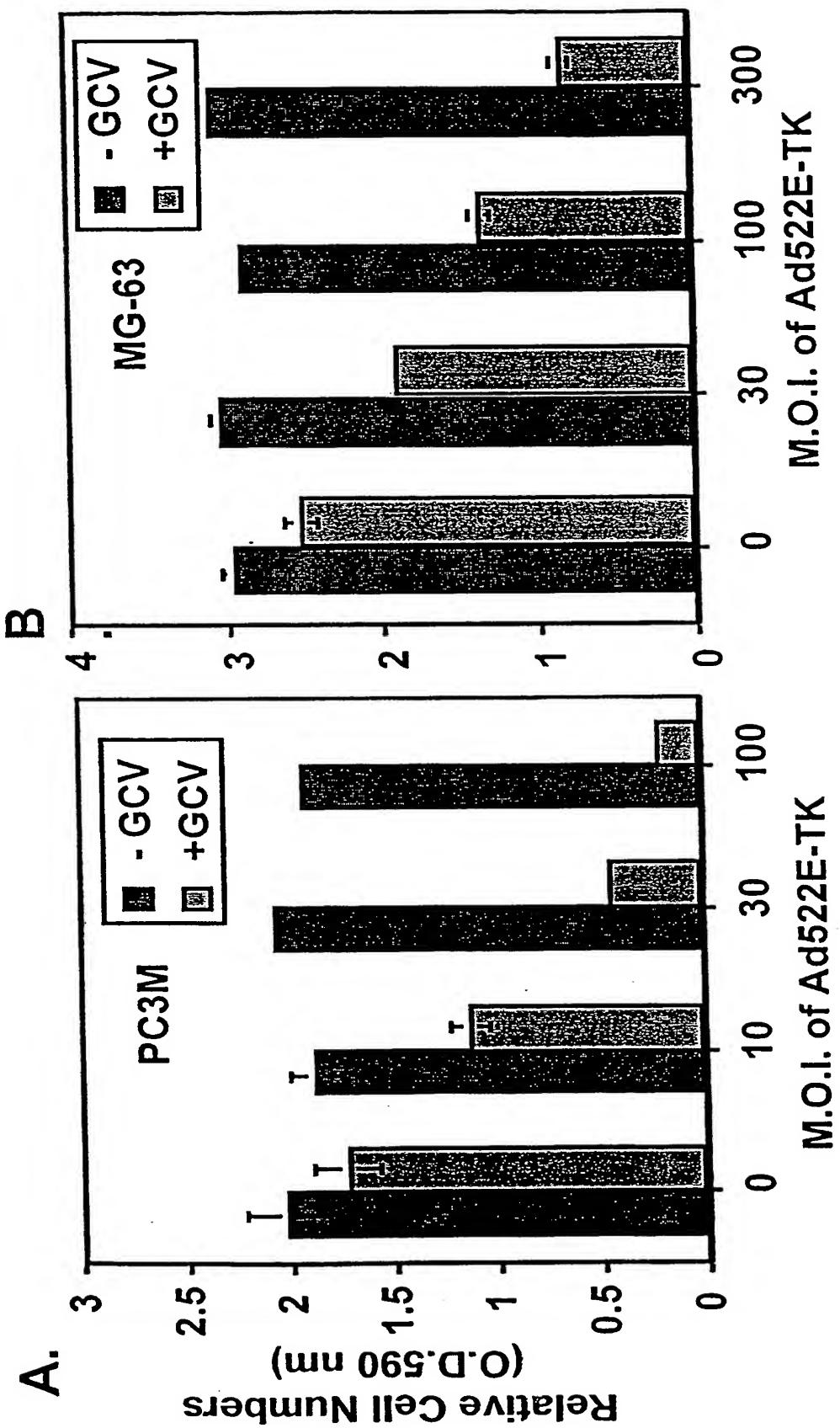
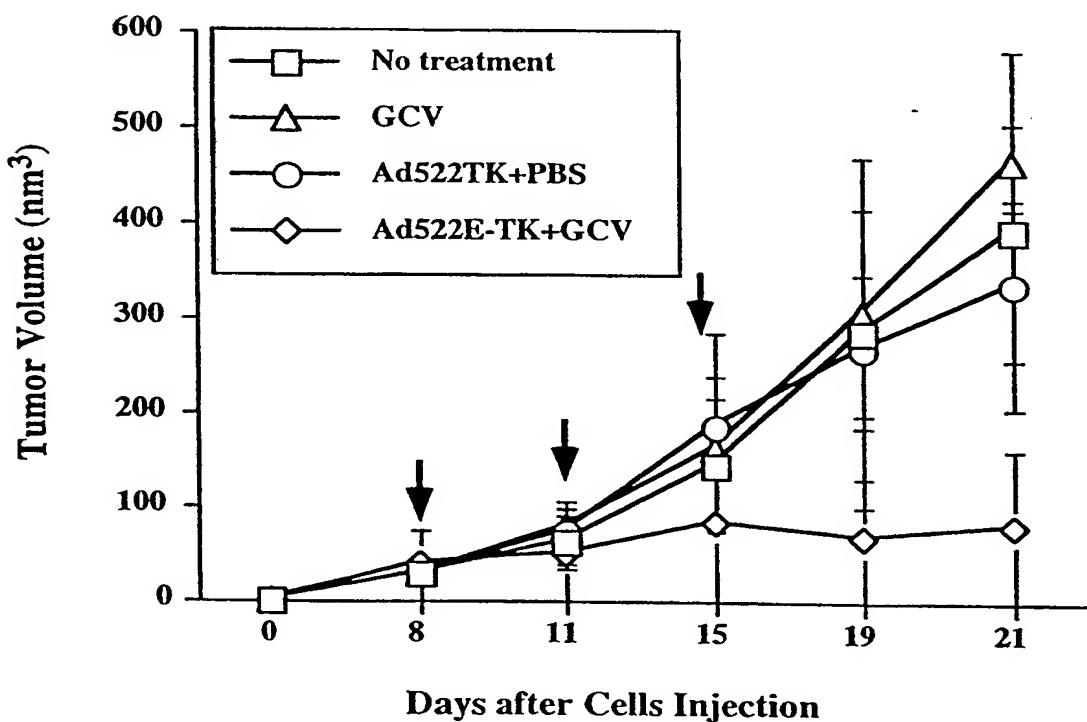
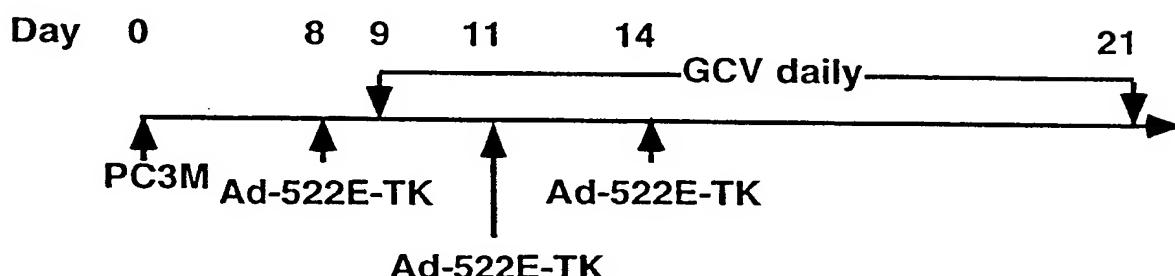


FIG.10 Cytotoxicity of Ad-522E-TK plus GCV in (A.) PC3M and (B.) MG63 cells. Cells were infected with indicating dose of Ad-522E-TK then cultured in the presence or absence of GCV for 5 days.

Figure 11: Sequences of human osteonectin promoter (hON) -522E

-550 ac tagtagcagg ttgttcttgtc
-500 tggcacgtgg taagtccctgg ccttgccttt gtggcaaata caacccctt
-450 gaattgcttg gccccttcga gcatttgccta atattagggaa ggactccctgt
-400 aaagctcaat ggttagaaga tcaagacact tggccctgg tctgccccctg
-350 gggccatgt ggttaattccct tgcatgtcc actgcctcaat tgccctctga
-300 acaagaaaga ggtcggtctg ggtcggtccct ccaggccctgt ccagccctgg
-250 cactctgtt gagtcgggtt aggcaagcagg cccggaaacag attagggcagg
-200 cagggttgg acgtttggta aggacagccc accggcaaaaaa gaggaggaaaa
-150 gaaatgaaag acagagacag -----
-100 gggaggaggaa gacaggaggaa ggagggaccac cgggtggatgg gggaggccgg
-50 cccaggcccaag agctctgagg tggtttcctgt tggctgtctc taaacccctc
+ 1 cacattcccg cggtccttca gactgcccgg agaggccgt ctggccctgtccg
+ 51 cctgcccggcc tg

Mice: athymic mice**tumor cells: PC3M****subcutaneous inoculation** **5×10^5 cells/site, 2 sites/ mouse, 4 mice/group****Virus: Ad-522E-TK****intratumoral injection** **2×10^9 pfu/site****GCV: 40 mg/kg****daily intraperitoneal (ip) injection for 2 weeks**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/14482

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01N 43/04

US CL : 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, medline caplus biosis embase, biotechds
WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,416,017 A (BURTON et al) 16 May 1995, see entire document especially abstract, column 6, lines 10-15, 41, and 42; paragraph bridging columns 10 and 11; column 11, lines 48-63; column 16, lines 29-48; column 24, lines 26-35; column 26, lines 58-65; column 29, lines 14-18, and 47-55.	1, 6-8, 11, 12, 16 -----
Y		13, 14
X	Database CAPLUS on STN, No. 1998:70287. DAMJONVSKI et al. 'Regulation of SPARC expression during early Xenopus development: Evolutionary divergence and conservation of DNA regulatory elements between amphibians and mammals'. Dev. Genes Evol. 1998 Vol. 207, No. 7, pages 453-461, abstract only.	1, 11, 13, 15, 16 -----
Y		6,7,8,12,14

 Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

02 OCTOBER 2000

Date of mailing of the international search report

18 OCT 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/14482

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANDERSON, W.F. Human Gene Therapy. Nature. 30 April 1998, Vol. 392, Suppl. pages 25-30, see entire document, especially page 25, column 2, first full paragraph; and page 30, column 1, third full paragraph, and fifth full paragraph.	2,3, 9, 10, 17-22, and 27-31
A	VERMA et al. Gene therapy- promises, problems, and prospects. Nature. 18 September 1997, Vol. 389, pages 239-242, see entire document, especially, page 239, second full paragraph.	2,3, 9, 10, 17-22, and 27-31

Form PCT/ISA/210 (continuation of second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/14482

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: 4, 5, AND 23-26 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
These claims required a search of a sequence listing, and no listing was submitted.

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

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